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13. ABSTRACT *(Maximum 200 words)*

Cancer of the breast and other tissues may be initiated or promoted by DNA adducts. Some of these adducts may be unknown at the present time, and differ from one individual to another both qualitatively and quantitatively. This makes it important to detect the entire spectrum of DNA adducts in breast and other tissues. Analytical methodology is not available for this at the present time, and there are practical or fundamental barriers against current methodology ever acquiring this capability. In this project new analytical methodology which promises to fill this gap has undergone significant development. The new methodology is based on fluorescence-dye postlabeling of DNA adducts, followed by capillary electrophoresis with laser-induced fluorescence detection. A more stable, second generation dye reagent was prepared and ultrapurified; immobilized metal ion affinity chromatography was set up to conveniently remove the dye after the labeling step; the methodology was applied in a preliminary way to the analysis of biological samples including human tissues; and it was found that the dye-labeled DNA adducts are readily detected by matrix assisted laser desorption ionization mass spectrometry.

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FOREWORD

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Roger Green 7/16/99

PI - Signature Date

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(5) INTRODUCTION

It is likely that DNA adducts initiate breast cancer and they may contribute as well to its progression. Current methods to measure DNA adducts in breast tissue are limited. The purpose of this project is to set up and apply powerful new analytical methodology for the detection of both known and unknown DNA adducts in human breast tissue. The hypothesis to be tested in this project is that nonpolar DNA adducts explain the epidemic of breast cancer. Because of the high content of fat in breast tissue, nonpolar chemicals may accumulate there. Risk factors may act by causing or promoting the occurrence of DNA adducts that in turn initiate or promote breast cancer. This role of risk factors potentially can be revealed by correlating them with DNA adducts in breast tissue. In turn, this may require comprehensive and accurate measurement of DNA adducts in such tissue. This cannot be done at the present time, a gap which our project is intended to fill.

The concept for our proposed new methodology for measuring DNA adducts is the same as ^{32}P-postlabeling, but the details are quite different. by changing the details, we intend to overcome the following limitations of ^{32}P-postlabeling: dependence of labeling on adduct structure, limited resolution, different conditions for different adducts, and the radiolabel barrier to analyzing the adducts by mass spectrometry.

In our new method, DNA is isolated from the tissue and digested to deoxynucleotides using conventional techniques. The deoxynucleotides are then separated by HPLC and fractions are collected. The deoxynucleotides in each fraction are fluorescence-labeled on their phosphate group with a BO-IMI fluorescent dye, followed by capillary electrophoresis with laser-induced fluorescence detection (CE-LIF). This dye specifically labels phosphomonoesters under aqueous conditions at room temperature.

(6) BODY

Introduction

We have organized this section of our final report according to our Statement of Work. As described below, we have been successful in accomplishing the Technical Objectives aside from some minor details, and even exceeded our Objectives in some respects. The overall scheme for our new methodology is shown in Fig. 1.

Task 1. Technical Objective (1). Capillary electrophoresis separations. Months 1-6

a. Losses of known polyaromatic and alkyl DNA adduct standards will be minimized in the overall analytical procedure.

We encountered a loss problem in one step of our overall procedure: the evaporation of the DNA adducts in the collected HPLC fraction. (The preceding HPLC step in the method provides partial resolution of the adducts, especially their separation from the normal nucleotides. In the step following this evaporation step, the adducts are labeled with the IMI dye.) It is essential to evaporate the HPLC fractions in order to set up a small reaction volume for the IMI2 labeling reaction, since this optimizes the labeling yield.

We began our evaluation of this problematic evaporation step by preparing benzo[a]pyrene diolepoxide DNA adducts of deoxyadenosine-5'-monophosphate (dAMP) and deoxyguanosine-5'-monophosphate (dGMP), as described in the manuscript of Appendix B, and evaporating a 1 mL volume in which the concentration of these compounds was 10^{-5} M. The loss of analyte upon redissolving, reaction with BO-IMI, and quantitation by CE-LIF was greater than 90%. However, if the same amount of these compounds was evaporated starting with a much smaller volume (e.g. 3 μL), the recovery was ≥10-fold higher. This meant that the adsorption losses were taking place onto the wall of the evaporation vial. We tested plastic, glass and silanized glass vials, and observed significant losses in each case. We assumed that the mechanisms were different, and decided to focus on the glass, using the recovery from a 3 μL volume in plastic as a reference (arbitrarily considered to be 100%). Our hypothesis for the loss mechanism on the glass was that the phosphate moiety of the nucleotides was binding to the glass. Based on this hypothesis, we introduced other phosphate species as carriers into the evaporation solution, selecting ones known to resist labeling by BO-IMI. All of the carrier phosphate compounds that we tested were successful in increasing the recovery of the DNA adducts, and two were considered to be most advantageous for this purpose : pyrophosphate and serine phosphate, since the recovery reached that of the reference sample. This is an interesting and important finding.

b. Interferences will be minimized in the overall analytical procedure.

We minimized interferences by introducing and optimizing the use of immobilized metal affinity chromatography (IMAC) as a way to remove residual IMI dye at the end of the labeling reaction. Conditions were optimized for this step, leading to loading and washing of the sample in 10% methanolic MES buffer. The

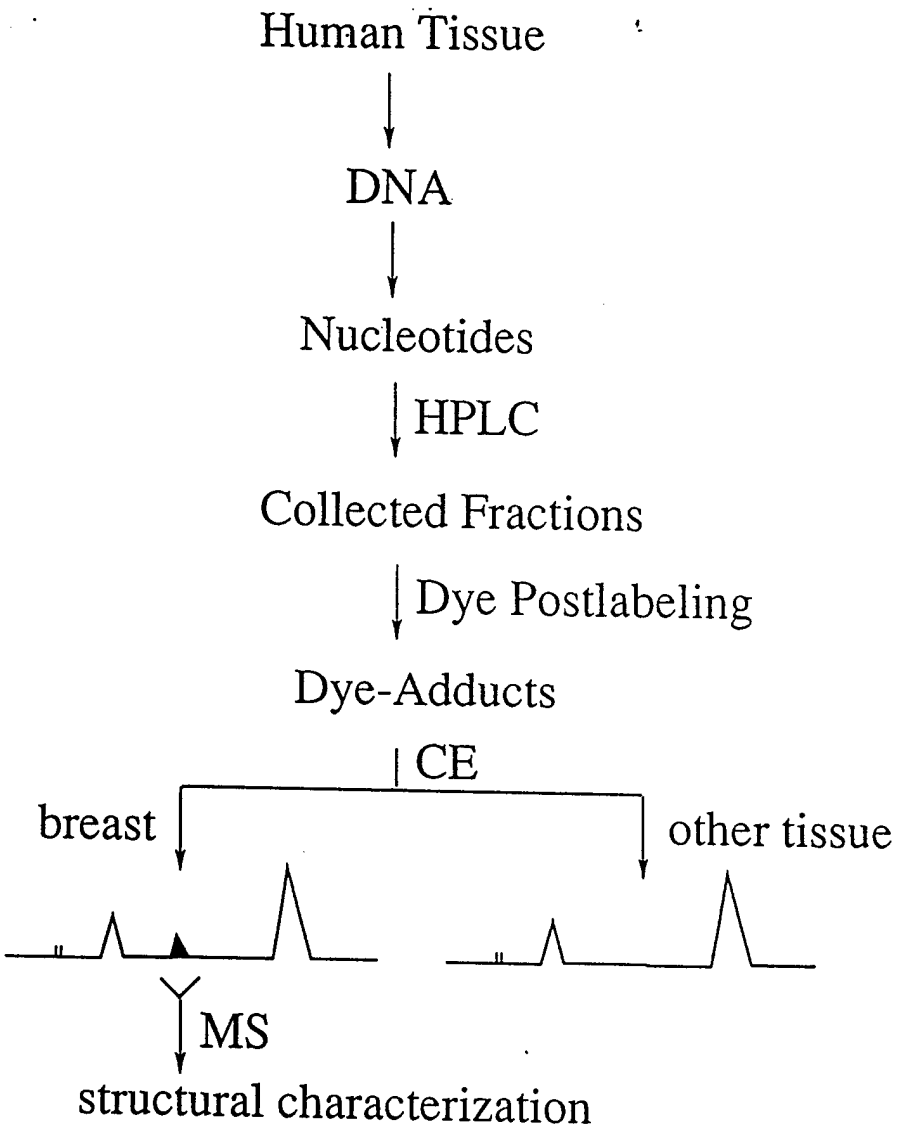

Fig. 1. Analytical scheme for the detection of DNA adducts in human tissue samples by fluorescence dye labeling/capillary electrophoresis. Scaled up DNA adducts can be subjected to mass spectrometry for some structural characterization.

IMI-labeled DNA adducts elute under these conditions in high yield, whereas the unreacted IMI dye, as intended, is retained.

To facilitate the purification of our BO-IMI dye, and also removal of excess dye after the labeling reaction, we have studied its separation, along with that of a precursor dye, BODIPY-hydrazide, by immobilized metal affinity chromatography (IMAC). The two compounds were separated on three forms of a Sepharose-IDA column: Cu(II), NI(II) and Zn(II). Whereas BO--IMI eluted first on the Cu(II) and NI(II) columns (a pH gradient from 7.0 to 2.0 was applied), it eluted last on the Zn(II) column. BO-Hz eluted from the Zn(II) column without displacing this metal. The explanation suggested for these results is that BODIPY hydrazide undergoes strong, bidendate binding only to the Cu(II) and Ni(II) columns. This work is described in the publication of Appendix A.

c. The selectivity of the separations by capillary electrophoresis will be optimized.

As shown in Fig. 1 of the Appendix B manuscript, we have set up two conditions for the CE separation. Not only do each of them provide high selectivity, but there is a good contrast in selectivity between them. This meets our goal of optimizing this parameter for the needs of this startup project. As seen, one of the conditions employs micelle electrokinetic chromatography (MEKC), while the other takes place in 10% methanol (non-MEKC). A high pH (10.6) is used in both cases, which is convenient.

d. The sensitivity will be optimized for the overall analytical procedure.

Three of the steps in the method needed to be optimized in order to optimize the sensitivity of the overall procedure, and this work has been accomplished. The first one is the IMI reaction. This was optimized by reducing the reaction volume to 3 μL in order to maintain a high concentration of the IMI dye. The second step is the removal of residual dye by IMAC (completed as discussed above) and the third is the recovery of adducts in the post-HPLC evaporation step (completed as discussed above).

Further, we studied the IMI labeling reaction in detail, as described in the Appendix C publication, and found out that the leash in BO-IMI, our first generation dye, was slightly susceptible to hydrolysis at the hydrazide linkage, leading to background noise. This was overcome by preparing a corresponding reagent (BO-IMI2) which contains an amide instead of a hydrazide group at this site, as described in the Appendix D publication. After conventional techniques failed to purify BO-IMI2 to a significant degree, which is essential for highest sensitivity, we developed a novel approach, involving submarine gel electrophoresis (usually employed for macromolecule separations). This raised the fluorescent purity level of BO-IMI2 to 99.9998%, as described in the manuscript of Appendix D.

While this successful work on BO-IMI2 was going on, in parallel we prepared a DBD-IMI dye and tested its sensitivity by CE-LIF. In this case a helium-cadmium laser was used. While the DBD dye was stable, unfortunately the sensitivity of DBD-IMI was 10-fold less than that of BO-IMI2, so we abandoned this approach. This work is described in the publication of Appendix E.

Task 2. Technical Objective (2). Known DNA adducts spiked into DNA samples. Months 6-12.

a. The detection of known polyaromatic and alkyl DNA adducts as standards will be achieved by BO-IMI labeling capillary electrophoresis.

As shown in Fig. 1 of the Appendix B manuscript, and already discussed above in another context, we did detect known polyaromatic DNA adducts by BO-IMI labeling capillary electrophoresis. This is described in more detail in the manuscript of Appendix B. Due to a cut at the outset in our budget, we lacked the resources to also detect alkyl DNA adducts, although it is now clear that this would be easy to accomplish since our method works for both polyaromatic-modified and ordinary nucleotides as described in the manuscript of Appendix B.

b. Such adducts will be detected starting with DNA samples (calf thymus DNA and tissue culture DNA) spiked with or containing these adducts.

This objective was reached as demonstrated by Figures 2 and 3. In Fig. 2A we show the detection of benzo[a]pyrene DNA adducts spiked into a sample of calf thymus DNA. The blank sample (lower electropherogram in Fig. 2A) was done in triplicate, and the three electropherograms for this (response scale expanded relative to Fig. 2A) are shown in Fig. 2B. As seen, the reproducibility of this triplicate data is excellent. In Fig. 3 we show the detection of such adducts formed by treating a culture of human cells with benzo[a]pyrene. The exposed cells for the latter analysis were obtained from William Thilly at MIT. Analyte identity and amount remain to be defined for the exposure sample in Fig. 3.

Task 3. Technical Objective (3). DNA in human breast tissue. Months 12-24.

a. Human breast, liver, lung, muscle, bowel and kidney tissues will be tested for known polyaromatic and alkyl DNA adducts.

We did not test these tissue for known DNA adducts. Since we had already accomplished the detection of polyaromatic DNA adducts in exposed cells, we bypassed it and went on to the next task.

b. Human breast, liver, lung, muscle, bowel and kidney tissues will be tested for unknown DNA adducts.

Autopsy sample of breast liver and lung were obtained from a 90-year old woman. The tissue samples were subjected to a phenol/chloroform procedure (that was furnished to us by Curtis Harris at NCI), yielding DNA (6 mg from 5 g liver, 2 mg from 5 g lung, and 0.8 mg from 15 g breast) which was digested to nucleotides with nuclease P1. Nonpolar DNA adducts were isolated by nonpolar extraction on C18-silica, labeled with BO-IMI2, and subjected to capillary electrophoresis with laser-induced fluorescence detection to yield the electropherograms shown in Fig. 4. Interference from phospholipids was ruled out by testing phospholipid standards and observing that they migrated later than the peaks observed here. Thus, the peaks apparently represent nonpolar DNA adducts in these samples. While the data is consistent with the hypothesis for this project, the data is preliminary, since blanks,

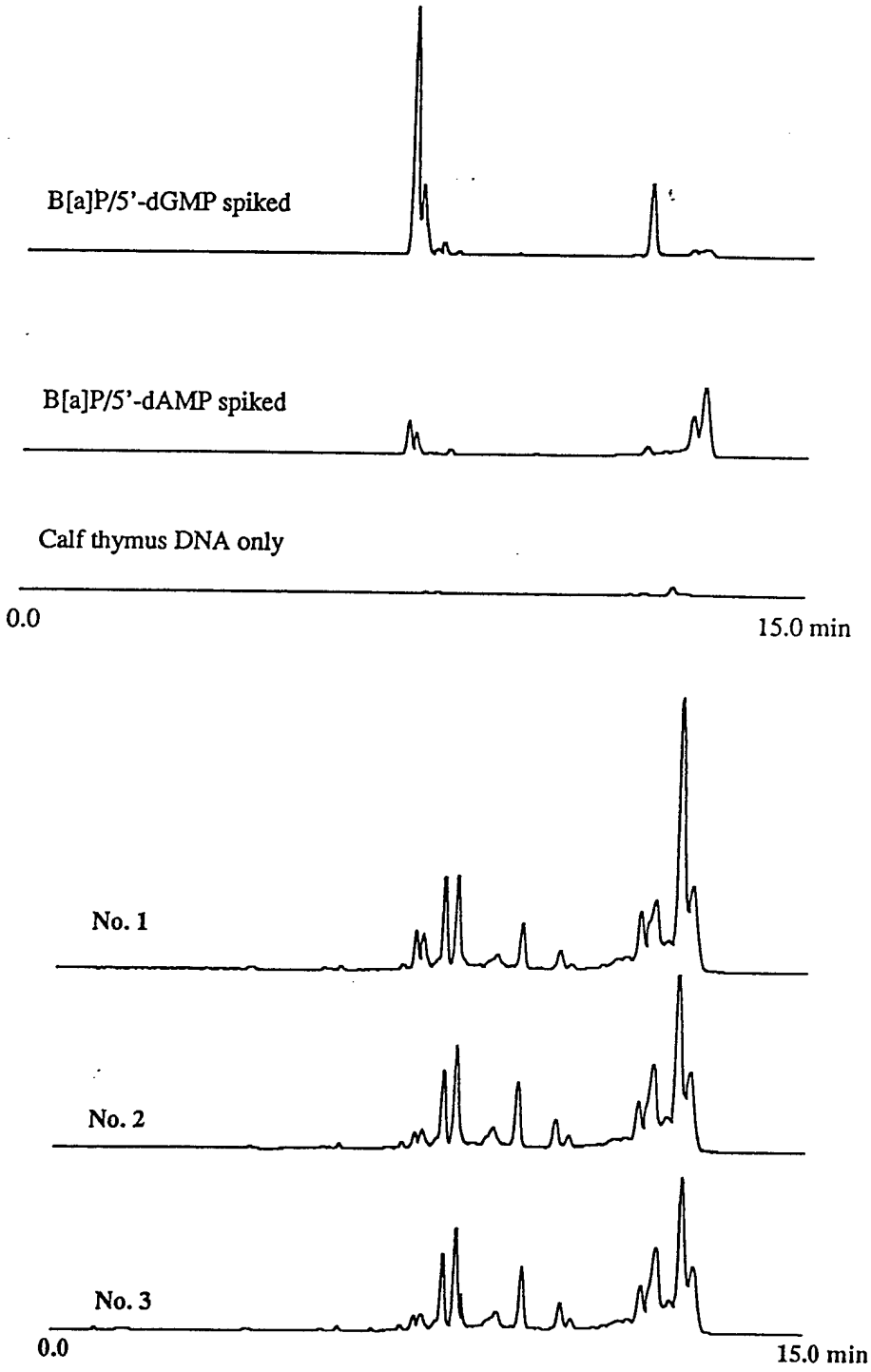

Fig. 2. Comparison by BO-IMI labeling/capillary electrophoresis of blank DNA and DNA spiked with benzo[a]pyrene DNA adducts. A. Calf thymus DNA (100 μg) was spiked with 5.2 nmol of benzo[a]pyrene-dAMP or 7.5 nmol of benzo[a]pyrene-dGMP adducts. After digestion with nuclease P1, extraction on an OASIS cartridge, labeling with BO-IMI2, and separation by immobilized metal ion affinity chromatography, 10 nL of the final volume of 100 μL was subjected to capillary electrophoresis with laser induced fluorescence detection. B. Expansion of the response scale for the bottom electropherogram in A: analysis (entire procedure) done in triplicate.

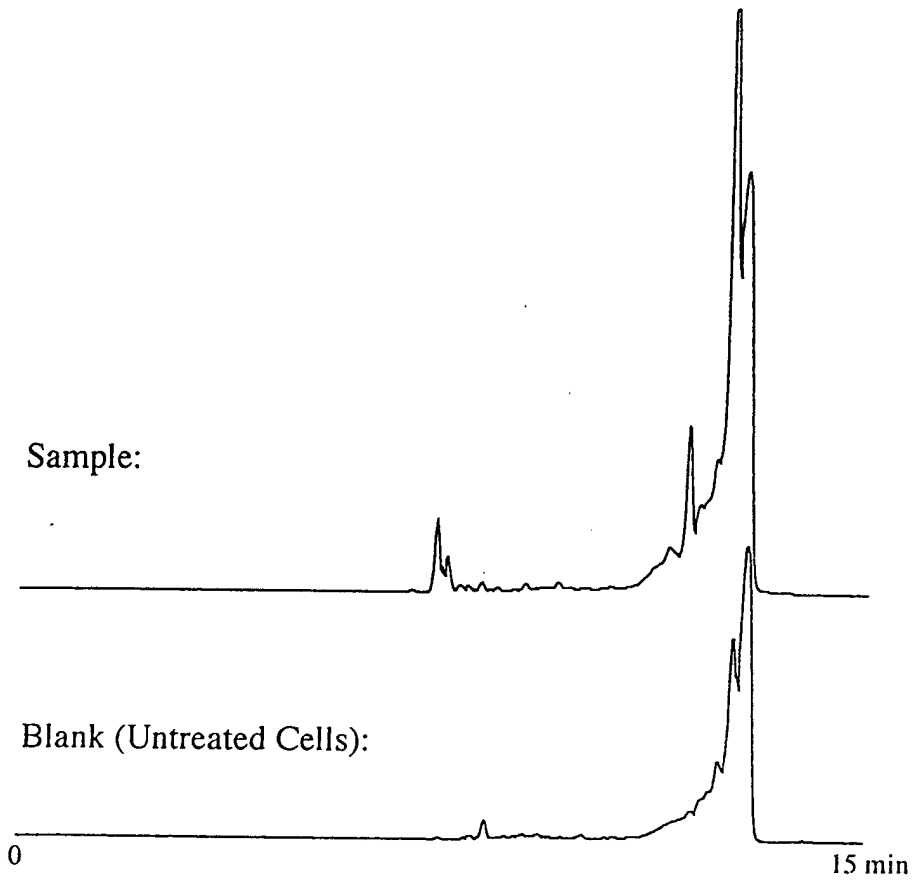

Fig. 3. Detection by BO-IMI2 labeling/capillary electrophoresis of DNA adducts in human lymphocytes exposed in cell culture to benzo[a]pyrene diolepoxide. The exposure was done by William Thilly and coworkers at MIT. A cell pellet was obtained from which the DNA was extracted using a Qiagen kit. The samples were then processed the same as described in Fig. 2A, leading to the electropherograms shown.

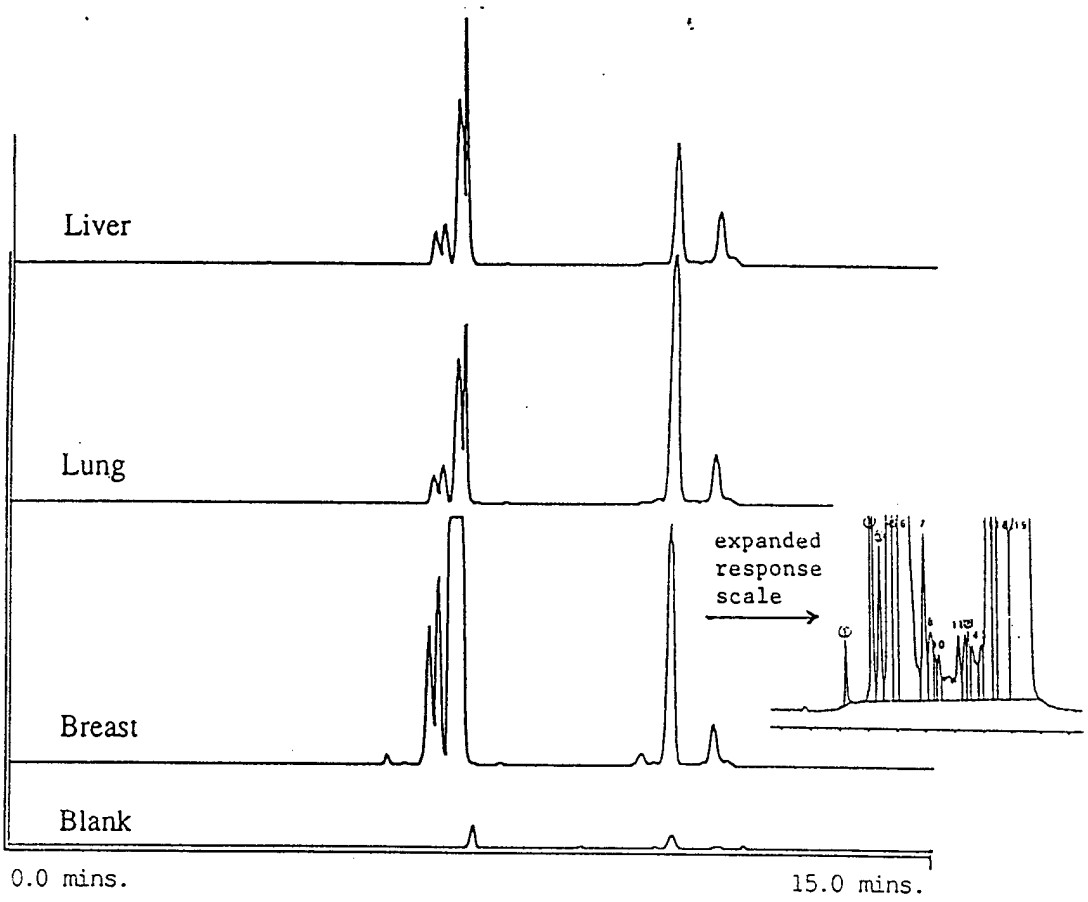

Fig. 4. Detection of IMI-reactive compounds in DNA samples isolated from autopsy specimens obtained for a 90-year-old woman. The tissue samples were subjected to a phenol/chloroform procedure (that was furnished to us by Curtis Harris at NCI), yielding DNA (6 mg from 5 g liver, 2 mg from 5 g lung, and 0.8 mg from 15 g breast) which was digested to nucleotides with nuclease P1. Nonpolar DNA adducts were isolated by nonpolar extraction on C18-silica, labeled with an IMI fluorescence dye using a method that is specific for phosphomonoesters, and subjected to capillary electrophoresis with laser-induced fluorescence detection to yield the electropherograms shown.

controls and repeat samples need to be tested. Unfortunately, at this point our budget was exhausted and the post-doc left for employment elsewhere.

(7) KEY RESEARCH ACCOMPLISHMENTS

1. Immobilized metal ion affinity chromatography of hydrazides.
2. Phosphate carriers enhance analyte recovery during evaporation.
3. Improved and ultra-purified second-generation IMI dye.
4. MALDI-TOF-MS of BO-IMI labeled DNA adducts.
5. Preliminary method application to human cell culture and tissue samples.

(8) REPORTABLE OUTCOMES

A. Manuscripts, abstracts, presentations.

1. Shen, X. and Giese, R.W., Hydrazide as a Ligand Moiety in Immobilized Metal Ion Affinity Chromatography: Separation of BO-IMI and BODIPY-Hydrazide, J. Chromatogr. A, 777, 261-265 (1997).

2. Lan, Z.H., Wang, P., and Giese, R.W., Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry of Deoxynucleotides Labeled with an IMI Dye, Rapid Comm. Mass Spectrom.,13, 1454-1457 (1999).

3. Wang, P. and Giese, R.W. Phosphate-Specific Fluorescence Labeling with BO-IMI: Reaction Details, J. Chromatogr., 809, 211-218 (1998).

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5. Lan, Z-H., Qian, X. and Giese, R.W. Preparation of an IMI Dye (Imidazole Functional Group) Containing a 4-(N,N dimethylaminosulfonyl)-2,1,3-Benzoxadiazole Fluorophore for Labeling of Phosphomonoesters, J. Chromatogr. A, 831, 325-330 (1999).

6. Lan, Z-H., Wang, P., Shen, X, Giese, R.W., Fluorescence/MS Detection of DNA Adducts, Proceedings of the 46th ASMS Conference on Mass Spectrometry and Allied Toppics, May 31-June 4, 1998, Orlando, FL, p. 417

7. Presentation: "Detection of DNA Adducts in Breast Tissue", Department of Defense Breast Cancer Research Meeting: Era of Hope, Washington, DC, October 1997 (Appendix G)

8. Presentation: "Trace Organic Analysis with Mass and Dye Tags," University of New Hampshire, October, 1998.

B. Patents and licenses

A patent was obtained by Northeastern University on this technology based on the data collected prior to this project.

C. Degrees obtained

Xiaohang Shen: Ph.D.

D. Development of cell lines

None.

E. Informatics

None.

F. Funding applied for based on work supported by this award

A three-year grant was obtained from NIEHS as a subcontract from MIT to apply the IMI technology to lung cancer.

G. Employment or research opportunities applied for an/or received on experiences/training supported by this award

Zhang-Hua Lan, a post-doc on this project, acquired skills in this project which enable him to work as an analytical chemist in the pharmaceutical or biotechnology industry.

(9) CONCLUSIONS

New methodology for detecting DNA adducts has been advanced significantly in this project. An improved dye reagent was prepared; a complete method has been set up including tuning of critical steps; and the method was applied, although only to a preliminary degree, to biological samples including human tissues. Beyond our original plans, we found that the dye-labeled DNA adducts have excellent detection properties by MALDI-MS: high sensitivity is achieved, in part the boost in mass from the dye label places the peaks in a relatively clean region of the mass spectrum.

So What? In spite of the obvious importance of measuring the entire spectrum of DNA adducts in a biological sample, practical analytical methodology for this purpose does not exist currently. ^{32}P-Postlabeling comes the closest to providing such detection, but the radioactivity and limited resolution make it impractical for this purpose. The fluorescence postlabeling capillary electrophoresis methodology that has been advanced in this project promises to fill this gap.

(10) BIBLIOGRAPHY OF PUBLICATIONS AND ABSTRACTS

1. Shen, X. and Giese, R.W., Hydrazide as a Ligand Moiety in Immobilized Metal Ion Affinity Chromatography: Separation of BO-IMI and BODIPY-Hydrazide, J. Chromatogr. A, 777, 261-265 (1997).

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8. Presentation: "Trace Organic Analysis with Mass and Dye Tags," University of New Hampshire, October, 1998.

(11) PERSONNEL RECEIVING PAY

Zhang-Hua Lan
Xiaohua Qian
Rong Jiang
Xiaohang Shen

(12) APPENDIX

A. Shen, X. and Giese, R.W., Hydrazide as a Ligand Moiety in Immobilized Metal Ion Affinity Chromatography: Separation of BO-IMI and BODIPY-Hydrazide, J. Chromatogr. A, 777, 261-265 (1997).

B. Lan, Z.H., Wang, P., and Giese, R.W., Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry of Deoxynucleotides Labeled with an IMI Dye, Rapid Comm. Mass Spectrom.,13, 1454-1457 (1999).

C. Wang, P. and Giese, R.W. Phosphate-Specific Fluorescence Labeling with BO-IMI: Reaction Details, J. Chromatogr., 809, 211-218 (1998).

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G. Presentation: "Detection of DNA Adducts in Breast Tissue", Department of Defense Breast Cancer Research Meeting: Era of Hope, Washington, DC, October 1997 (Appendix G)

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JOURNAL OF CHROMATOGRAPHY A

Hydrazide as a ligand moiety in immobilized metal ion affinity chromatography
Separation of BO–IMI and BODIPY–hydrazide

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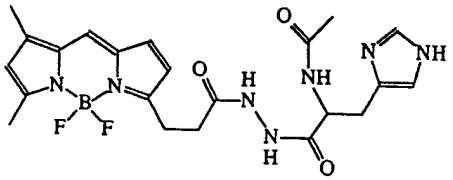
Abstract

BODIPY hydrazide (BO–HZ, a commercially available fluorescent dye) and BO–IMI (obtained by coupling the hydrazide moiety of BODIPY to the carboxyl group of *N*-acetylhistidine) were separated on three forms of a Sepharose–iminodiacetic acid column: Cu(II), Ni(II) and Zn(II). Whereas BO–IMI eluted first on the Cu(II) and Ni(II) columns (a pH gradient from 7.0 to 2.0 was applied), it eluted last on the Zn(II) column. BO–HZ eluted from the Zn(II) column without displacing this metal. The explanation suggested for these results is that BODIPY hydrazide undergoes strong, bidendate binding only to the Cu(II) and Ni(II) columns. © 1997 Elsevier Science B.V.

Keywords: Detection, electrophoresis; Hydrazides; BO–IMI; BODIPY–hydrazide

1. Introduction

A fluorescent reagent called 'BO–IMI' (structure is shown below) is available for covalent labeling of phosphate monoesters [1,2].



BO-IMI

In this labeling reaction, the phosphate moiety of the target substance is activated with a water soluble carbodiimide for attack by BO–IMI. This yields a phosphoroimidazolide product in which the imidazole moiety of BO–IMI has replaced an oxygen atom of the phosphate group. The reaction takes place under aqueous conditions at room temperature and is specific for phosphomonoesters. While carboxylic acids are also activated by the carbodiimide, they do not form a hydrolytically-stable conjugate with BO–IMI. To date the reaction has been applied to nucleotides [1], some phosphate metabolites [1], and the phosphoprotein pepsin [2]. In all cases, the BO–IMI-labeled analyte was detected by capillary electrophoresis with laser-induced fluorescence detection (CE–LIF).

Here we report the separation of BO-IMI and BODIPY-hydrazide (BO-HZ), a synthetic precursor of BO–IMI, by immobilized metal ion affinity

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chromatography (IMAC) on a Sepharose–iminodiacetic acid (IDA) column. We were motivated to conduct this study for two reasons. First, it was hoped that IMAC might provide an efficient way to remove any traces of BO–HZ and some other impurities from BO–IMI at the end of the synthetic procedure for this latter compound. This could minimize the background of interfering peaks when a trace analyte is detected via labeling with BO–IMI. Second, IMAC potentially could serve as well to remove residual BO–IMI from BO–IMI-labeled analyte at the conclusion of the labeling reaction.

IMAC was introduced by Porath [3], has been reviewed [4–6], and has been studied in detail more recently (e.g., [7–10]). Most applications have involved peptides or proteins, where the side chain of histidine and the *N*-terminal NH_2-CHR-CONH group can be the two predominant ligands. Nevertheless, many factors can play a role such as type of metal, degree of metal loading, pH, and the overall structural features of the peptide or protein, potentially including a contribution from its other nucleophilic groups [10]. For proteins engineered to possess a hexa-histidine *N*-terminus, a Ni(II)-nitrilotriacetic acid form of IMAC, introduced by Hochuli et al. [11,12], has become important, e.g. [13]. The behavior of a hydrazide in IMAC apparently has not been reported before.

2. Experimental

2.1. Materials and chemicals

Chelating Sepharose Fast Flow (Sepharose-IDA) was purchased from Pharmacia (Uppsala, Sweden). Zinc sulfate and copper sulfate were obtained from Mallinckrodt (St. Louis, MO, USA). Nickel sulfate and sodium acetate were from Aldrich (Milwaukee, WI, USA). Ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA) was obtained from Fisher Scientific (Pittsburgh, PA, USA). 4,4-Difluoro-5,7-dimethyl-4-bora -3a,4a-diaza -3-indacenepropionyl hydrazide (BODIPY hydrazide, BO–HZ) was obtained from Molecular Probes (Eugene, OR, USA). BO–IMI was synthesized as described [1]. The acetate buffer was 0.1 *M* sodium acetate, pH 4.0, and the phosphate buffer was 0.1 *M* sodium phosphate, pH 7.0.

2.2. Separation of BO–IMI and BO–HZ by IMAC

Sepharose–IDA in ethanol (1.5 ml) was packed into a 10 ml disposable pipet plugged with glass wool. After the column was washed with 20 ml of distilled water, 10 ml of 0.2 M metal sulfate (copper, nickel or zinc) were applied. The unbound metal ions were washed off with 20 ml each of acetate and distilled water, and the column was equilibrated with 20 ml of phosphate. A 100 μl sample which contained 10 μg of BO–IMI and 10 μg of BO–HZ in phosphate was loaded onto the column and eluted with a stepwise pH gradient, starting from phosphate and followed by acetate–HCl buffers: pH 6, 5, 4, 3 and 2. The fractions were tested by CE–LIF.

2.3. Capillary electrophoresis

The capillary electrophoresis system, which is fitted with a laser-induced fluorescence detector (argon ion laser; 488 nm excitation, 520 nm detection) has been described [1]. The separation was achieved in a 65 cm (40 cm to detector)×75 μm I.D. capillary at 25 kV with anodic siphoning injection (column elevated 5 cm for 20 s). The running buffer is 0.01 *M* 2-(N-morpholino)ethanesulfonic acid, 0.0035 *M* tris(hydroxymethyl)aminomethane, 10% acetonitrile, pH 6.2.

2.4. Preparation of zinc-free solvents and tubes

Water and buffers for the following experiment involving zinc measurements were made metal-free by extraction with dithizone in chloroform as described [14]. Residual chloroform was removed by N_2 bubbling. Nitric acid (15%) soaking followed by washing with metal-free water was used to make the test tubes metal-free.

2.5. BO-HZ on Zn(II)-IMAC followed by Zn measurement

Sepharose-IDA gel (1.5 ml) was packed into a 5 ml disposable pipet and washed with 20 ml water. After adding 2 ml of 0.2 *M* $ZnSO_4$, 20 ml of acetate

and 50 ml of phosphate were applied to wash out unbound zinc. BO–HZ (15 μg) in 0.5 ml of phosphate was loaded onto the column and eluted with 25 ml of phosphate. Fractions were collected and analyzed for zinc content by atomic absorption spectroscopy.

3. Results and discussion

Target substances bound on an IMAC column typically are eluted by a gradient to a lower pH, or by the addition of imidazole, ammonium acetate, or a chelating agent like EDTA. For our purposes, involving preparative or analytical purification, a pH gradient was most attractive. Based on the extensive literature concerning IMAC of histidine-containing substances, we started the separation at pH 7.0 in 0.1 *M* sodium phosphate buffer, and selected to first test a Ni(II) form of IMAC.

Neither BO–IMI nor BO–HZ migrated on the Sepharose–IDA at pH 7.0 (visual observation of the yellow–green band of sample on the top of the column bed, which can be enhanced by exposure to a UV lamp, providing 365 nm), so we began a stepwise pH gradient employing 0.1 *M* sodium acetate buffer (HCl treated). The separation was monitored more quantitatively by collecting fractions and testing them off-line by CE–LIF (data not shown). These conditions nicely resolve the two compounds by Ni(II)–IMAC, with BO–IMI eluting much earlier than BO–HZ, as shown in Fig. 1A.

Similar testing of Cu(II) and Zn(II) forms of Sepharose–IDA gave the chromatograms displayed in Fig. 1B and 1C, respectively. As seen, the two compounds are separated under both of these conditions as well, but with a change in the order of retention on the Zn(II) column. We did not optimize any of the separations, since they were all adequate for our purposes. These chromatograms show that the decreasing order of retention is Cu(II)>Ni(II)> Zn(II) for BO–IMI, and for BO–HZ it is Cu(II)~ Ni(II)>Zn(II). When Belew et al. [15] tested several proteins on a TSK polymer IDA column, eluting with a linear gradient of imidazole, they similarly observed the retention order Cu(II)>Ni(II)>Zn(II). However, Porath reported the order Cu(II)>Zn(II)> Ni(II) for serum proteins on a Sepharose–IDA

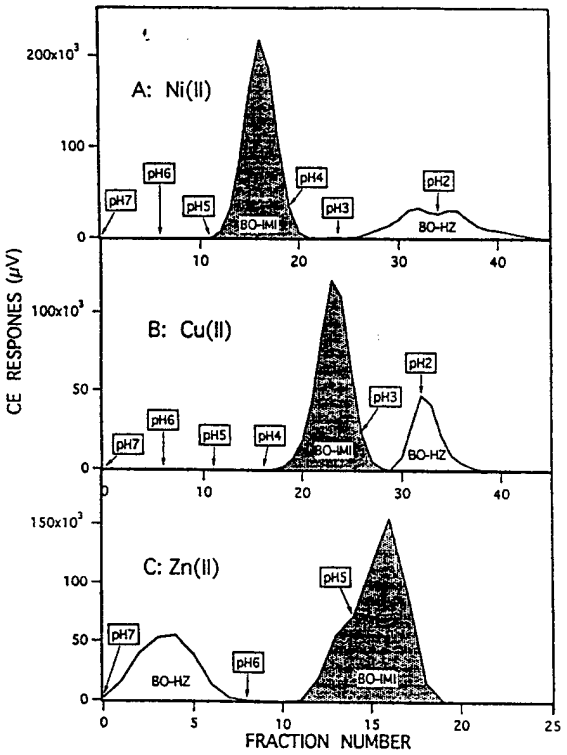
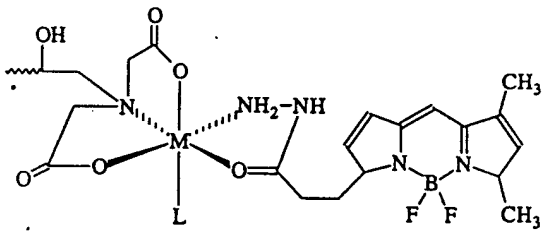


Fig. 1. IMAC chromatograms obtained by loading a Sepharose-IDA column in the Ni, Cu, or Zu form, as indicated, with a solution of BO-IMI and BO-HZ at pH 7.0, and eluting with stepwise changes to lower pH while monitoring the colored bands visually, and also collecting 2 ml fractions for measurement of BO-IMI and BO-HZ by CE-LIF.

column [3], as did Andersson and Sulkowski [16]. Anyway, the complexity of proteins and the variation in analytes and conditions among these studies makes it difficult to make meaningful comparisons.

We wish to speculate, in part, on the mechanisms producing the separations shown in Fig. 1. All three metal ions are medium–soft in their polarizability [5], so this concept does not seem to be helpful. It is known that hydrazides tend to bind as bidentates to metal ions [17–19], whereas imidazole acts as a monodentate ligand, so this could explain the stronger retention of BO–HZ than BO–IMI on the Ni(II) and Cu(II) packings. This speculation is represented in Fig. 2. Similarly, Hansen et al. [20] have pointed out that the *N*-terminal end of a peptide, in principle, may bind as a bidentate (α-



M = Cu (II); Ni (II)
L = arbitrary ligand

Fig. 2. Postulated bidendate binding of BO-HZ to the Ni(II) and Cu(II) forms of Sepharose-IDA. L=arbitrary ligand.

amino and nearby carbonyl) to Sepharose-IDA–Cu(II).

The weak retention of the BO–HZ on the Zn(II) column could be due either to a very weak (e.g., from monodentate binding) or strong binding of the Zn(II) by BO–HZ. In the latter case, BO–HZ would have eluted rapidly by stripping the Zn(II) from the IDA chelate, a general mechanism that has been called 'metal ion transfer' for IMAC [8]. We ruled out the latter mechanism by observing no displacement of zinc by BO–HZ from the Zn(II) column. Others have observed that peptides lacking histidine fail to retain on a Sepharose–IDA–Zn(II) column, even when they possess an *N*–terminal α-amino group [16,20].

The strong binding of a hydrazide on a Sepharose–IDA–Ni(II) or –Cu(II) column might be exploited. For example, many substances contain one or more carboxylic acid groups, and this group can be converted easily to a hydrazide, potentially enabling the substance then to bind reversibly on such a packing. If successful, this would expand the variety of extrinsic groups useful as 'affinity handles' in IMAC, which presently comprise multi-histidine groups [11–13] and certain di- or tripeptide sequences containing a terminal histidine [21,22]. A second, general suggestion is that a simple hydrazide like acetylhydrazide might be employed as an alternative eluent for an IMAC column. A final suggestion for future experiments is to test the behavior of hydrazides on other versions of IMAC, such as *tris*(carboxymethyl)ethylenediamine (TED; [5]) and nitrilotriacetic acid (NTA; [11]) columns.

4. Conclusion

BO–IMI and BO–HZ, which are imidazole and hydrazide species, respectively, can be separated readily on a Sepharose-IDA column. When the column is in the Ni(II) or Cu(II) form, BO–IMI elutes first, whereas BO–HZ elutes first on the Zn(II) form of the column. Potentially the usefulness of IMAC columns can be broadened by taking advantage of hydrazide as a ligand.

Acknowledgments

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RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. **13**, 1454–1457 (1999)

Appendix B

Matrix-assisted Laser Desorption/Ionization Mass Spectrometry of Deoxynucleotides Labeled with an IMI Dye

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The four major deoxynucleotides of DNA, and adduct mixtures resulting from separate reactions of 5′-dAMP and 5′-dGMP with benzo[*a*]pyrene diolepoxide (BPDE), were analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) after labeling of their phosphate group with an IMI dye. The latter reagent comprises an imidazole functional group attached to a BODIPY (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene) fluorophore. Good sensitivity was observed in the detection of the IMI-labeled products by MALDI-MS: 300–500 fmol in the laser spot (1% of the 30–50 pmol sample on the target) gave a signal-to-noise (S/N) of ≥30 from 20–30 superimposed laser shots. The BPDE reaction products, after the IMI labeling, were also subjected to capillary electrophoresis with laser-induced fluorescence detection, which revealed a complex mixture of products. Overall the results encourage the further development of this 'IMI-postlabeling' methodology as an alternative to ^{32}P-postlabeling for the detection of DNA adducts.

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DNA adducts, consisting of sites on chromosomal DNA where covalent damage has taken place, are an important class of trace analytes. This is particularly so for human samples, where the measurement of DNA adducts is of interest to define human exposure to carcinogens. For nearly every exposure classified by the International Agency for Cancer Research as a human carcinogen, the agent is known to damage DNA, either by forming characteristic DNA adducts, or by contributing to general damage such as oxidation.[1]

For the measurement of unknown DNA adducts in human samples, a technique called '^{32}P-postlabeling' is widely used, as has been reviewed.[2] In this overall method, DNA is first isolated from a biological sample and digested enzymatically to nucleotides or dinucleotides. Once some of the adducts in the sample have been enriched (e.g. bulky, nonpolar adducts are extracted into butanol), they are labeled radioenzymatically with ^{32}P derived from [^{32}P]ATP. In the final step the adducts are detected by radioactivity counting after separation by thin layer chromatography (TLC) or high performance liquid chromatography (HPLC). The technique can also be used to detect known DNA adducts.

While ^{32}P-postlabeling is an important technique for detecting DNA adducts, it has some limitations.[2] The yield of the radioenzymatic reaction can vary widely for different DNA adducts. The technique does not provide structural information about an unknown DNA adduct except possibly when an unknown TLC spot or HPLC peak co-elutes with a known adduct. The resolution of the overall method is held back by the ^{32}P, since this restricts sample handling, e.g. one hesitates to use capillary electrophoresis or multi-dimensional HPLC to improve the separation of the adducts. In principle ^{32}P-labeled DNA adducts could be scaled up for analysis by mass spectrometry, but few investigators are willing to contaminate their instrument in this way.

To overcome these problems, we are developing a new method, 'IMI-postlabeling,' for detecting DNA adducts.[3–7] The concept for this technique is the same as that of ^{32}P-postlabeling, except that: (1) the labeling reagent is a fluorescent IMI dye (so called because imidazole is the functional group for labeling); (2) the labeling reaction is conducted chemically rather than enzymatically (a carbodiimide is used to activate the phosphomonoester of the DNA adduct for coupling to the IMI dye); (3) capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) is used to detect the IMI-labeled DNA adducts; and (4), as demonstrated here, IMI-labeling facilitates detection of DNA adducts by MALDI-MS.

EXPERIMENTAL

Materials

2-[*N*-Morpholino]ethanesulfonic acid (MES) was obtained from Sigma (St. Louis, MO, USA), and 0.1 N NaOH was used to adjust a 10 mM solution of it to pH 7.0. Benzo[*a*]pyrene-*trans*-7,8-dihydrodiol-9,10-epoxide (anti) (BPDE) was from the NCI Chemical-Carcinogen Repository (Midwest Research Institute, Kansas City, MO, USA). Propanesulfonic acid silica, from J.T. Baker Inc. (Phillips-

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CCC 0951–4198/99/141454–04 $17.50

burg, NJ, USA), was converted to the NH_4^+ form by treatment with NH_4OH to pH 9.6, followed by washing with water. IMI2 was prepared as a methanolic stock solution as described.[7] 2′, 4′, 6′-Trihydroxyacetophenone (THAP) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) were from Aldrich (Milwaukee, WI, USA).

BPDE-DNA adducts

BPDE adducts of 5′-dAMP and 5′-dGMP were prepared as described by Szeliga[8] based on a procedure by Peltonen *et al.*[9] One mL of 40 mg/mL deoxynucleotide in 0.10 M sodium citrate, pH 5.0, and 0.5 mL of 1 mg/mL BPDE in tetrahydrofuran, each at room temperature, were mixed quickly. After storage in an ice bath overnight, the sample was extracted with 3 × 1 mL each of water-saturated ethyl acetate (to remove residual BPDE and its hydrolysis products) and then diethyl ether (the latter to remove ethyl acetate). After nitrogen bubbling for 5 min to remove the ether, the sample was loaded onto a 12 cm^3 C18-silica (2g) Sep-Pak Vac cartridge (Waters Associates, Milford, MA) that had been washed with 30 mL each of methanol and then citrate buffer. After the cartridge had been washed with 30 mL of water, it was eluted with 5 × 3 mL of methanol. Only the third fraction was retained, since it contained most of the product, which was estimated to be about 2.6×10^{-4} M based on a UV measurement at 345 nm, using the molar extinction coefficient of benzo[*a*]pyrene-r-7,t-8,9-c-10-tetrahydrotetrol in tetrahydrofuran (Data sheet for this compound from Midwest Research Institute). This sample was kept at −70 °C.

IMI labeling

Thirty μL each of the following were combined: (1) 0.01 M deoxynucleotide in 0.1 M pH 7.0 MES buffer, (2) 0.1 M EDC in the same buffer, and (3) 1.7×10^4 M IMI2 in methanol. (The limiting amount of IMI2 is coupled quantitatively to the nucleotide under these conditions, as determined by reaction monitoring with CE-LIF). The volume was reduced to about 40 μL on a SpeedVac concentrator (Savant Instruments, Holbrook, NY, USA). After standing overnight in the dark at room temperature, the sample was then ready for analysis by MALDI-MS (see below). IMI-labeled BPDE-DNA adducts were prepared similarly, except they were desalted prior to MALDI-MS by treatment of 40 μL of sample with 10 μL of propanesulfonic acid silica (NH_4^+ form), followed by stirring (rotation by hand) for 1 min.

CE-LIF

This was conducted as described,[6] with hydrostatic injection at the anode end and detection with an argon-ion laser. The buffer for micellar electrokinetic chromatography (MEKC) was 10 mM pH 10.6 sodium carbonate, 50 mM sodium dodecylsulphate (SDS), and the SDS was omitted for non-MEKC.

MALDI-MS

Five μL of 0.5 M THAP in methanol, prepared on the day of use, was combined with 5 μL of essentially aqueous, IMI-labeled nucleotide and 1 μL (containing 30 pmol of IMI-labeled BPDE-nucleotide, or 50 pmol of IMI-labeled nucleotide) was loaded on the stainless steel probe to make a spot 2 mm in diameter. After the sample had been allowed to dry in air (≤10 min), a MALDI mass spectrum was obtained in negative ion mode by firing 20–30 superimposed laser shots (100–200 μm in diameter) on a Proflex instrument (Bruker Daltonics, Billerica, MA, USA) with delayed extraction. This corresponded to irradiating 1% of the sample assuming that the laser spot was 200 μm.

RESULTS AND DISCUSSION

To provide standard DNA adducts for testing, we reacted benzo[*a*]pyrene diolepoxide with 5′-dAMP and 5′-dGMP

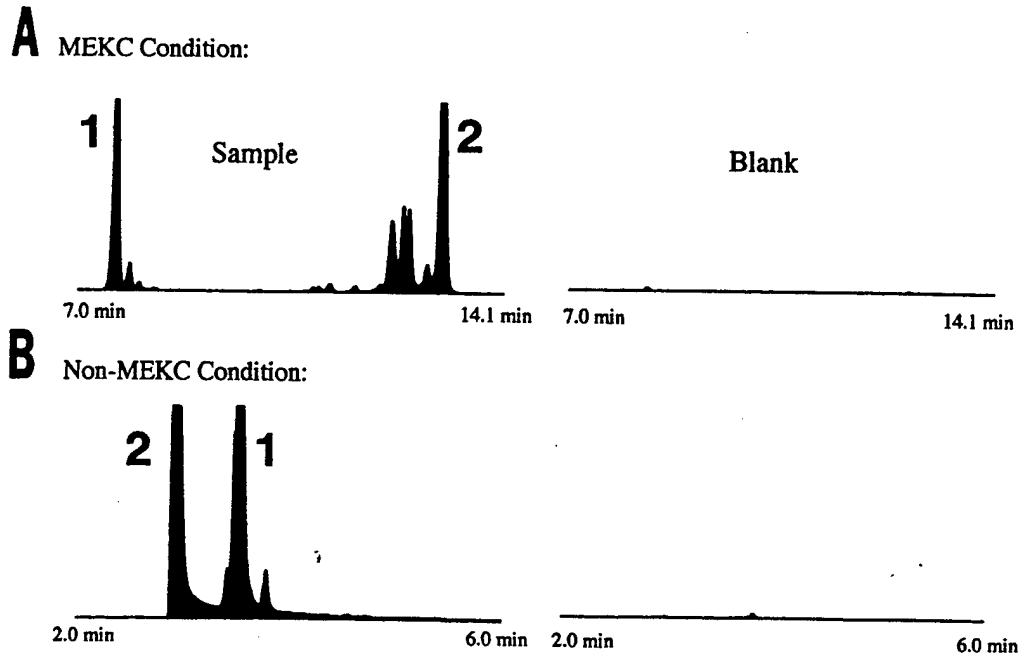

Figure 1. Electropherograms of products from the reaction of 5′-dAMP with benzo[*a*]pyrene-r-7, t-8-dihydrodiol-t-9,10 epoxide (±), (anti).

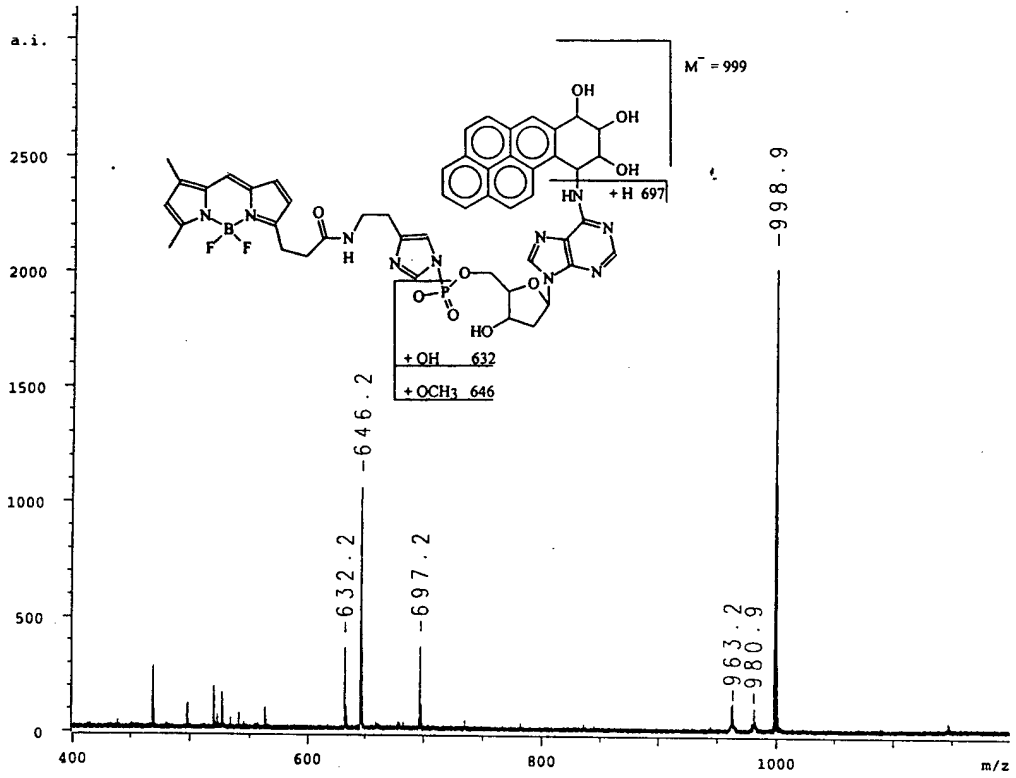

Figure 2. Detection of an IMI2-labeled DNA adduct standard by MALDI-MS: 20 superimposed shots; 1% of the 30-pmol sample applied to the target is in the laser beam; nitrogen laser; THAP matrix.

essentially as described,[9] including the use of C18 silica with water washing and methanol elution to enrich the adducts. After the sample had been labeled with IMI2, it was analyzed by CE-LIF. Comparable results from this analysis were obtained for the dAMP and dGMP products; only those from the former compound are shown in Fig. 1. As seen, the sample was analyzed under both MEKC and non-MEKC conditions (electropherograms in Fig. 1(a) and 1(b), respectively). No peaks appeared under either conditions for the blank samples (IMI reaction conducted on a blank sample) as also shown in this figure.

We did not truly identify any of the peaks in the electropherograms, but a tentative assignment can be made for the two major peaks based on the known reaction that we conducted,[8,9] the electrophoretic behavior of these peaks, and the mass spectral data that we will shortly present. Peak 1, which migrates as an anion in both electropherograms, apparently is the known BPDE-10-N^6-5′-dAMP adduct (structure shown in Fig. 2; prepared before as a nucleoside[10]), and peak 2, which migrates as a neutral, apparently is the related, known BPDE-10-N7-5′-dAMP adduct (also prepared before as a nucleoside[10]).

Under MEKC conditions (Fig. 1(a)), the BPDE-10-N^6-5′-dAMP adduct migrates relatively rapidly to the detector (carried along by the dominant electroosmotic flow) due to its negative charge, which minimizes its interaction with the negatively charged, high-mobility micelles. In contrast, the neutral BPDE-10-N7-dAMP adduct interacts more strongly with the micelles and thereby migrates slowly to the detector. There is a reversal of migration order of these peaks in the change from MEKC to non-MEKC conditions (Fig. 1(a) to 1(b)) since the migration of the N7 adduct as a neutral brings it to the detector before the negatively charged N-adduct under non-MEKC conditions.

The entire sample was subjected to MALDI-MS, leading to the mass spectrum shown in Fig. 2. As seen, the spectrum has been interpreted as coming from the N^6-adduct. This is arbitrary, since we also could have assigned the ions listed to an [M-H] species of the N7-adduct. Residual methanol in the matrix leads to the ion at 646.2 u. The ions at 981 and 963 u are not assigned since they correspond both to loss of one and two water molecules, respectively, and also to replacement of one and two atoms of F by H. (At high pH, it is known that these F atoms are replaced by OH[6].)

The main point in regard to Fig. 2 is that the IMI-labeled adduct mixture, in spite of the acid-lability of the phosphoimidazolide linkage,[3] gives rise to an intense, reproducible signal: 1% of the 30 pmol of sample applied to the target is within the laser beam; the S/N is about 60; and a uniform response is observed when different zones of the sample are laser desorbed. In part this good sensitivity derives from the boost in mass of the nucleotide adduct by the attached IMI dye, which helps to bring the molecular ion into a relatively noise-free region of the spectrum.

Equivalent results in terms of sensitivity and types of fragments are obtained with the corresponding sample from the reaction of 5′-dGMP with BPDE followed by IMI labeling: M^- at 1015.95 with fragment peaks at 998.3, 980.4, 713.7, 648.8 and 662.9 u; data not shown. Slightly less sensitivity for M^- (S/N $\geq$30) was observed when we similarly tested the four, ordinary nucleotides of DNA (data not shown), although they were not desalted. Thus the presence of a bulky chemical on the nucleobase is not essential for a good response from an IMI-labeled deoxynucleotide by MALDI-MS.

CONCLUSIONS

The results here encourage further development of IMI-postlabeling for the analysis of known and unknown DNA adducts in human and other biological samples. While the highest sensitivity may result when the IMI-labeled adducts are detected by CE-LIF, detection of such adducts after scale-up (larger biological samples) by MALDI-MS opens up the opportunity to confirm known adducts, and to partly characterize unknown adducts, helping to overcome some of the shortcomings when DNA adducts are detected by ^{32}P-postlabeling.

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JOURNAL OF CHROMATOGRAPHY A

Phosphate-specific fluorescence labeling with BO-IMI: reaction details

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Abstract

Previously we reported that BO-IMI, a reagent which contains a BODIPY fluorophore linked to an imidazole group, can be used to covalently label a phosphomonoester in a single step under aqueous conditions [P. Wang, R.W. Giese, Anal. Chem. 65 (1993) 3518]. The reaction was conducted in the presence of a water-soluble carbodiimide 1-ethyl-3-(3′-N,N′-dimethylaminopropyl)carbodiimide [EDC] to activate the phosphomonoester, and the coupling took place onto both the N1 and N3 imidazole nitrogens of BO-IMI. Whether the two BO-IMI-phosphomonoester regioisomers migrated separately or together during capillary electrophoresis depended on the pH, due to a difference in their pK_a values. Since then, we have studied the reaction in more detail leading to the information reported here. First, we have learned that the regioisomer ratio changes during the course of the reaction, and found that the mechanism involves both spontaneous and BO-IMI-catalyzed hydrolysis of the less stable isomer. Second, there is a background reaction in which BO-IMI becomes attached to EDC. Third, the BO-IMI-phosphomonoester product (a mixture of two isomers), that is observed by capillary electrophoresis at an alkaline pH, is found to no longer contain the two fluorine atoms present in the starting BO-IMI reagent. This is because they are replaced by hydroxy groups at high pH. Finally, an event was discovered which complicates the detection of less than about 60 fmol of a phosphomonoester with BO-IMI: hydrolysis of a tiny fraction of the BO-IMI takes place during the coupling reaction, which leads to chemical noise in the capillary electropherogram.

Keywords: Derivatization, electrophoresis; BO-IMI; Nucleotides

1. Introduction

Recently we introduced a new reagent and technique which achieves phosphate-specific fluorescence labeling under aqueous conditions [1,2]. The new reagent is "BO-IMI", a fluorescent derivative of N-acetyl-histidine. In our technique the phosphate moiety of a target compound is activated with a water soluble carbodiimide for single-step coupling to the imidazole moiety of BO-IMI. The method is phosphate specific since carboxylic acids are labeled only transiently by BO-IMI during the reaction. Due to our interest in detecting a class of trace analytes called DNA adducts, we primarily tested nucleotides. Diluted standards of BO-IMI labeled nucleotides were detected at the low attomole level (polarity-switching injection of 4.4 μl containing 2.2 amol of each BO-IMI-nucleotide) by capillary electrophoresis with laser-induced fluorescence detection (CE-LIF)

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[1]. We have also specifically labeled a phosphate group on pepsin, a phosphoprotein, with BO-IMI [3]. Our basic technique for labeling a target phosphate compound with BO-IMI consists of three steps: (1) BO-IMI coupling at pH 6; (2) filtration through a cation exchange silica packing at pH 7.0; and (3) CE-LIF at pH 10.4. The filtration step removes the residual BO-IMI and water soluble carbodiimide along with related decomposition products. A high pH is typically used for the subsequent separation by capillary electrophoresis in order to enhance selectivity, since some nucleobases have pK_a values in the vicinity of this pH.

Here we report the results of experiments which lead to a more detailed understanding of our new reagent and technique. This work was motivated primarily by our interest in extending the method to labeling of trace quantities of nucleotides. Thus far we have detected 60 femtomoles of dAMP, but have encountered chemical noise at this level, partly because of a defect in the current reagent.

2. Experimental

2.1. Materials

BO-IMI[1],C8-[N-acetyl-N-(2-fluorenyl)]amino-5′-dGMP [4] (C8-AAF-5′-dGMP), C8-oxo-5′-dAMP [5], and C8-benzyloxy-5′-dAMP [5] were prepared as described. 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl hydrazide (BODIPY FL C_3 hydrazide) was from Molecular Probes (Eugene, OR, USA). Sodium tetraborate (BORAX), tris[hydroxymethyl]-aminomethane (TRIS), 1-ethyl-3-(3′-N,N-dimethylaminopropyl)carbodiimide (EDC), 2-(N-morpholino)-ethanesulfonic acid (MES), 5′-dAMP, 5′-dCMP, 5′-dGMP, and 5′-TMP were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC-grade acetonitrile, methanol and propyl sulfonic acid silica (40 μm, 60 Å) were from J.T. Baker Inc. (Phillipsburg, NJ, USA). Ammonium acetate (NH_4Ac) was from Fluka (Buchs, Switzerland). 5′-pCAAAGCTTG (a DNA oligomer) was from Oligos Etc. Inc. (Wilsonville, OR, USA). Microcentrifuge tubes (1.5 ml, 05-407-10, Fisher Scientific, Pittsburgh, PA, USA) were used for the BO-IMI reactions.

2.1.1. Buffers

Buffer A (pH 6.0): 0.5 ml of 0.2 *M* MES, 0.38 ml of 0.1 *M* NaOH, and 9.1 ml of water.

Buffer B (pH 6.0): 2.0 ml of 0.2 *M* MES, 0.28 ml of 0.5 *M* TRIS, 4.0 ml methanol, and 34 ml of water.

Buffer C (pH 8.7): 0.8 ml of 0.5 *M* boric acid, 0.8 ml of 0.5 *M* TRIS, 4 ml acetonitrile, and 34.4 ml of water.

Buffer D (pH 10.4): 2.0 ml of 0.05 *M* BORAX, 1.8 ml of 0.1 *M* NaOH, 5 ml of acetonitrile, and 41.2 ml of water.

2.1.2. Equipment

A home-built capillary electrophoresis (CE) apparatus with laser-induced fluorescence detection (Ar ion laser with excitation at 488 nm) was used [6]. The CE unit was interfaced to a Macintosh Centris 610 computer through DYNAMAX MacIntegrator I (Rainin Instrument Co., Inc., MA, USA). One of the contact input ports was used to trigger the data acquisition, and one of the contact outputs was used to interlock and trigger the regulated high-voltage d.c. power supply (Glassman High Voltage Inc., NJ, USA). CE was performed in a 70 cm long fused-silica capillary (75 μm I.D.) with the detection window 45 cm from the injection end. Samples were injected hydrodynamically: anode end 5 cm higher for 20 s (about 10 nl).

2.2. Fluoride measurement of hydrolyzed BODIPY hydrazide

An Orion 501 digital ionalyzer and an Orion F^- ion-selective electrode (Orion Research Inc., Cambridge, MA, USA) were used for F^- measurement. Fluoride standard solutions, which gave a linear calibration curve, were prepared by weighing KF (MCB, Norwood, OH, USA), dissolving it to 0.1 *M* in 0.1 *M* potassium phosphate, pH 6.5, and making dilutions in this buffer to 10^{-5} *M* fluoride. After 1.0 ml of a 8.9×10^{-4} *M* solution of BODIPY hydrazide in 0.01 *M* NaOH was kept for 0.5 h at room temperature, it was diluted with 9 ml of the 0.1 *M* phosphate buffer and tested, revealing a fluoride concentration of 1.8×10^{-4} *M* (1.8×10^{-3} *M* in the original solution).

2.3. Cation exchange filtration of a BO-IMI/nucleotide reaction mixture

BO-IMI (1.2×10^{-2} *M*), 5′-dAMP (1.2×10^{-1} *M*), and EDC (1.2×10^{-1} *M*) in buffer A, 10 μl each, were briefly mixed and then kept at room temperature in the dark for 15 min. The starting BO-IMI was removed by loading the reaction mixture onto a Pasteur pipet column containing ~140 mg propyl sulfonic acid silica retained on glass wool, and eluting (with air pressure from a rubber bulb, including a terminating flow of air through the column) with 2×0.3 ml of buffer A. Before this separation was performed, bulk cation exchanger in this buffer was adjusted to pH 6 with 0.1 *M* NaOH, and, after the column was prepared, it was washed with 10 ml of buffer A.

2.4. Detection of 60 and 600 fmol of 5′-dAMP

BO-IMI (1.2×10^{-2} *M*), 5′-dAMP, and EDC (9×10^{-2} *M*), 1 μl each in buffer A, were mixed, and the resulting solution was kept dark at room temperature for 2 h. After the reaction mixture was diluted with 0.5 ml of pH 7.0 0.01 *M* NH_4Ac (pH adjusted with NH_4OH), it was loaded onto a cartridge column (see below). A rubber bulb was employed to push all the liquid out of the cartridge into a collection vial. A second 0.5 ml NH_4Ac was applied and similarly pushed into the vial. The collected solution was evaporated to dryness in a Speed-Vac (Savant Instruments). Fifty μl of buffer D was added followed after 30 min by injection into the CE capillary. To prepare the cartridge column, bulk cation exchanger in 0.01 *M* NH_4Ac was adjusted to pH 7.0 with 0.1 *M* NaOH, and loaded (140 mg) into a pasteur pipet plugged with glass wool followed by washing with 10 ml of pH 7.0 0.01 *M* NH_4Ac.

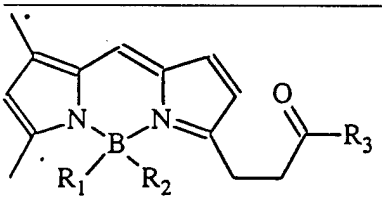
3. Results and discussion

Previously we pointed out that both the N1 and N3 nitrogens of the imidazole moiety of BO-IMI (**1**; the structures of our compounds are presented in Table 1) react with a phosphomonoester [1]. For 5′-dAMP and 3′-dAMP (and the other corresponding mononucleotides that were studied), the ratio of the major (**3**) to minor (**2**) product was 97:3 and 98:2, respectively. It was assumed (but not proved) that this product ratio arose kinetically as a consequence of steric effects, tentatively making **3**=N1 regioisomer and **2**=N3 regioisomer for BO-IMI-5′-dAMP (see Table 1 for a definition of the N1 and N3 positions). To obtain this data, we conducted the labeling reaction at pH 6.0 for 3 h, and then subjected the product mixture to capillary electrophoresis at the same pH. This separated the pair of isomeric products, apparently due to a difference in the pK_a values of their imidazole moieties in this pH region. For example, the imidazole moiety of guanosine 5′-phosphoimidazolide has a pK_a of 6.07 at 37°C [7]. At an elevated pH (≥8.7 was examined) the isomeric products comigrated, conveniently allowing a single peak to be observed for each target phosphate compound that was tested.

When the reaction mixture (at pH 6.0) is directly examined by CE-LIF after a shorter time period, e.g., 0.3 h, as shown in Fig. 1A, a higher ratio of **2** relative to **3** is seen relative to what is observed after 3 h (Fig. 1B). Thus there is a shift with time from a kinetically to a more thermodynamically-controlled product mixture. Further observations, about to be described, suggest that the primary mechanism for this shift involves preferential hydrolysis at pH 6.0 of the less stable isomer (**2**), which releases the 5′-dAMP for a second round of coupling to BO-IMI (excess EDC, a water soluble carbodiimide, is present).

We subjected a reaction mixture (containing a 10-fold molar excess of 5′-dAMP over BO-IMI) after 15 min to cation exchange filtration, which removed the residual EDC (but apparently not any preformed EDC-5′-dAMP, since it lacks a net charge). The collected sample was divided into two parts (A and B). Part A was immediately treated with an amount of BO-IMI equivalent to what was present initially; this adjustment was done without changing the pH. Buffer A was added to part B to keep the volume the same. The samples were stored at room temperature in the dark, while aliquots of each were tested periodically by CE to monitor the ratio of isomeric products. Thus we were testing the effect of BO-IMI on the ratio of **2** to **3** in the absence of intact EDC, to avoid the complication of hydrolyzed **2** or **3**

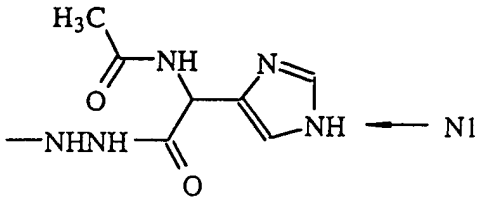
Table 1
Structures of BODIPY derivatives



1=BO-IMI

	R_1	R_2	R_3
1,1′	F, OH	F, OH	X
2,2′	F, OH	F, OH	X-5′-dAMP[a]
3,3′	F, OH	F, OH	X-5′-dAMP[a]
4,4′	F, OH	F, OH	$NHNH_2$
5,5′	F, OH	F, OH	see Fig. 5
6′	OH	OH	$NHNHCOCH_3$ [b]
7′	OH	OH	X-C8-benzyloxy-5′-dAMP[c]
8′	OH	OH	X-C8-AAF-5′-dGMP[c]
9′	OH	OH	X-C8-oxo-5′-dAMP[c]
10′	OH	OH	OH
11′	OH	OH	X-pCAAGCTTG

X =



[a] Phosphoimidazolides where one is N1 and one is N3 with respect to the imidazole moiety. Which isomer is the major product is unknown, however.
[b] Postulated structure.
[c] Mixture of N1 and N3 phosphoimidazolides with respect to the imidazole moiety.

re-reacting with EDC. After 20 h, the pH of the A and B parts were 6.23 and 6.40, respectively.

As the absolute peak area for **2** decreased progressively in B (the part not supplemented with BO-IMI) throughout this storage period (first order kinetics; $t_{1/2}$=2.3 h), the peak for BO-IMI increased correspondingly, while that for **3** decreased to a lesser degree. (Previously we determined that the half-life for **3** at pH 6.0 is 19.7 h [1]). In part A (supplemented with BO-IMI), fourfold less **2**, and slightly more **3** (about 10%), was present at the first time point (1.1 h after the filtration step) than in part B containing no added BO-IMI. Apparently the added BO-IMI in part A was doing two things: reacting with EDC-5′-dAMP (which accounts for the initial, slight increase of **3** in A) and also catalyzing the hydrolysis of **2** (which both lowers the pH and the yield of **2** in A relative to what happens in B). Thus, both spontaneous and BO-IMI-catalyzed hydrolysis of the less stable isomer **2** in the ordinary reaction mixture (where excess EDC is present) appear to drive its conversion, via reactivation with EDC, to **3**. In the ordinary reaction (which is usually conducted for 3 h), this thereby leads to the 97:3 product ratio of **3:2**. Other known catalysts for hydrolysis of a phosphoimidazolide of a nucleotide include both $H_2PO_4^-$ and HPO_4^{2-} [7].

Assuming, as before, that **2** is the N3 isomer, then why does this isomer hydrolyze more rapidly at pH 6.0? It is known that a phosphoimidazolide hydrolyzes more rapidly when the imidazole moiety is protonated [7]. We speculate that the postulated N3 isomer hydrolyzes faster since protonation at its free N1 site can be stabilized by solvation with little interference from the remote C4 alkyl group, in contrast to the behavior of the N1 isomer.

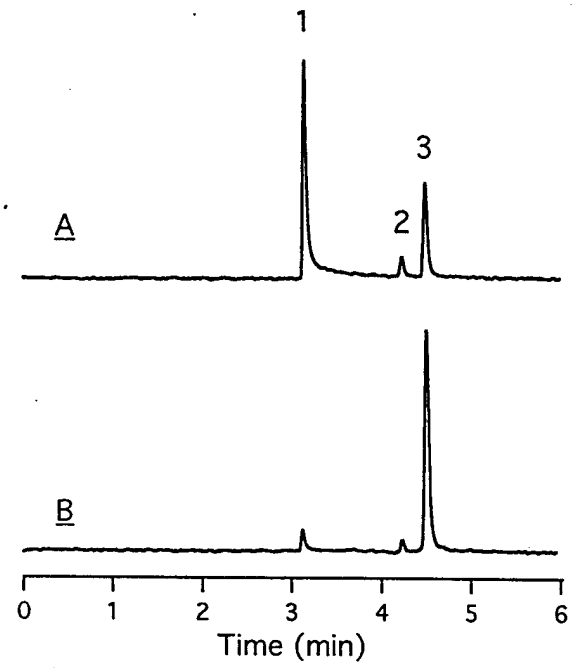


Fig. 1. Electropherograms at pH 6.0 (buffer B) showing two BO-IMI-5′-dAMP isomers (**2** and **3**) as a function of reaction time, which was either 0.3 (A) or 3.0 h (B). Peak **1** is BO-IMI. Sample preparation: BO-IMI (2.4×10^{-2} *M*), dAMP (2.4×10^{-2} *M*), and EDC (1.8×10^{-1} *M*) in buffer A, 10 μl each, were combined.

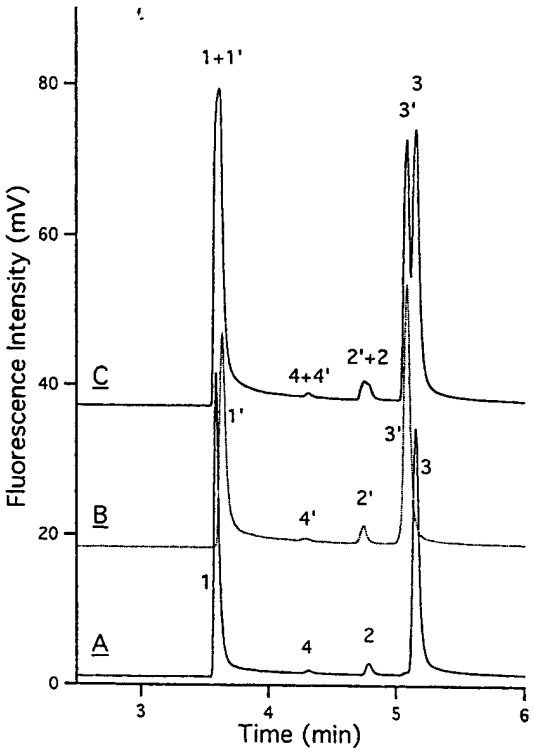


Fig. 2. Effect of pH on BO-IMI-5′-dAMP. (A) Fresh reaction mixture of BO-IMI, dAMP and EDC. (B) Sample after incubation in Buffer D (pH 10.4) for 0.5 h at room temperature. (C) Coinjection of B and C. Peaks: **1**=BO-IMI; **2** and **3**=BO-IMI-5′-dAMP; **4**=BODIPY FL C_3 hydrazide. **1′**, **2′**, **3′**, **4′** are the corresponding dihydroxy compounds. The separation was done at pH 6.0 (buffer B).

The electropherogram shown in Fig. 2A basically corresponds to those shown in Fig. 1 except that a small amount of BODIPY FL C_3 hydrazide, **4**, is present as a neutral marker. When the pH of this reaction mixture is raised to 10.4 and then, one hour later, returned to pH 6.0, the electropherogram shown in Fig. 2B is observed. Coinjecting the two reaction mixtures gives the electropherogram shown in Fig. 2C. Considering the relative peak positions as well as areas, it certainly appears that compounds **2** and **3** are converted to **2′** and **3′**, respectively, by the intermediate exposure to high pH. (Shortly we will also discuss compounds **1′** and **4′**).

What happens at an elevated pH is that the two fluorine atoms attached to the boron are replaced by hydroxy groups. This was demonstrated in two ways. First, a fluoride ion selective electrode was used to determine that two equivalents of fluoride are formed when hydrazide **4**, as a pure sample, is exposed to alkaline pH. Second, appropriate protonated molecule and fragment ions were observed for compound **4′** by fast atom bombardment mass spectrometry (data not shown). Compound **4** and its hydrolysis product **4′** comigrate at both pH 6.0 and 10.4 by CE (data not shown), demonstrating the neutrality of the dihydroxy product **4′**. Also seen in Fig. 2 is a peak for **1′**, the dihydroxy version of BO-IMI.

Some of the BO-IMI forms a conjugate with EDC during the course of the reaction. To confirm the identity of this side product, **5**, a sample of a reaction mixture was subjected to fast atom bombardment mass spectrometry, leading to the mass spectrum shown in Fig. 3. No incubation at pH 10.4 was done for the latter sample, so the species detected was the difluoro compound. The peak at *m/z* 174 apparently arises from reaction of the released *m/z* 156 moiety,

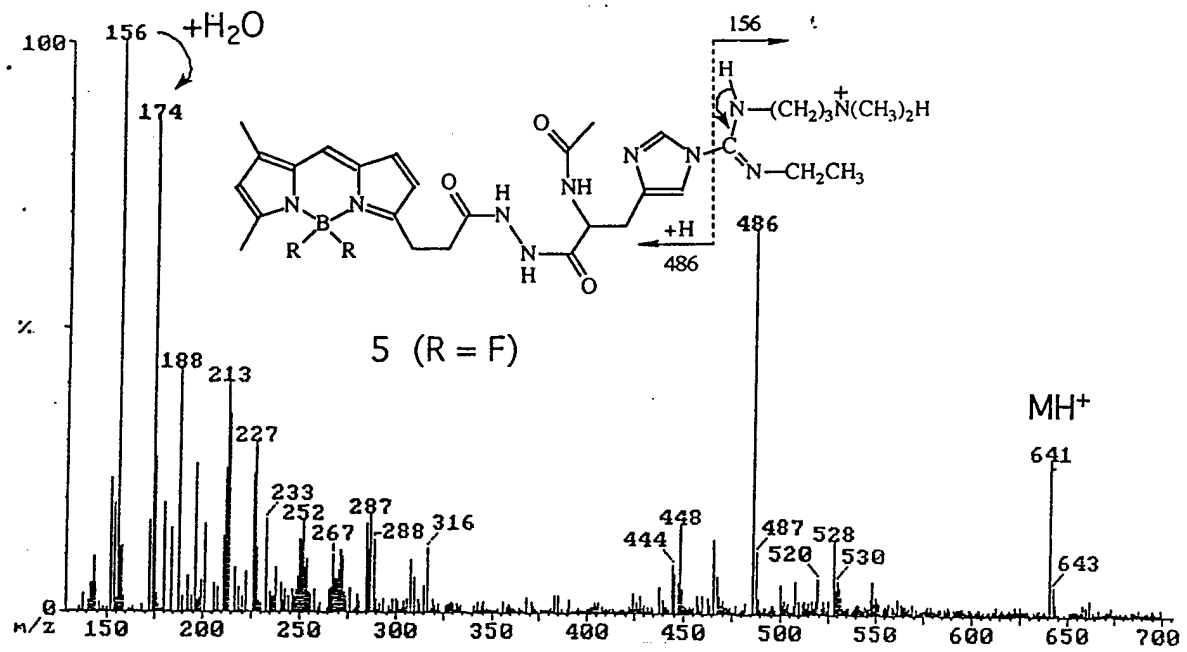


Fig. 3. Mass spectrum (capillary liquid chromatography continuous-flow fast atom bombardment) of BO-IMI-EDC, **5**. The solvent was 1% glycerol in 0.01 *M* ammonium acetate, and the instrumentation and technique were described before [9].

a carbodiimide, with water to form the corresponding urea, as indicated in the figure. Compound **5** partly reforms **1** when exposed to 0.1 *M* HCl for 5 h, consistent with the known hydrolytic instability of an imidazole–carbodiimide conjugate at low pH [8].

Both compounds **5′** and **1′** migrate as anions at pH 10.4. There is no known pK_a for imidazole in this pH region. BODIPY FL C_3 hydrazide and its dihydroxy analog have essentially the same migration times at this pH, so the charge cannot arise from the dihydroxyboron moiety. Thus some ionization of the dicarbohydrazide component of **5′** and **1′** must be taking place. To support this hypothesis, we acetylated BODIPY FL C_3 hydrazide in acetonitrile with acetic anhydride, and observed that the product, **6′**, unlike dihydroxy BODIPY hydrazide, **4′**, also migrates as a negatively charged species at pH 10.4. The alkaline pK_a values of dihydroxy BO-IMI and **6′** were found to be 10.0 and 10.8, respectively, by monitoring their electrophoretic mobility relative to that of BODIPY hydrazide (neutral marker of electroendosmosis) as a function of pH (data not shown).

In Fig. 4 is shown the separation by CE of BO-IMI conjugates of normal deoxynucleotides, two DNA adducts (compounds **8** and **9**) and also a synthetic precursor, **7**, for the preparation of **9**. The inset in this figure shows the electropherogram of a BO-IMI labeled DNA oligomer. This latter result was reported before [1], but the electropherogram was not shown. Each conjugate gives a single peak since the N1 and N3 isomers for each one comigrate at alkaline pH.

With the existing reagent and conditions we achieved the detection of 60 femtomoles of 5′-dAMP as shown by the electropherogram in Fig. 5B. Corresponding electropherograms for the detection of 600 femtomoles of 5′-dAMP, and for a blank reaction, are shown in Fig. 5A,C, respectively. While these samples were cation-exchange filtered prior to the CE separation, a peak for dihydroxy BO-IMI (**1′**) is observed. Apparently there are two origins of this peak: hydrolysis of some **2′** and **3′** (especially of the less stable **2′**), and incomplete removal of BO-IMI (a limited amount of cation-exchange packing was employed). The presence of a significant peak for compound **10′**, dihydroxy BODIPY FL C_3 carboxylic acid, reveals some hydrolytic instability of BO-IMI under the reaction conditions, along with the

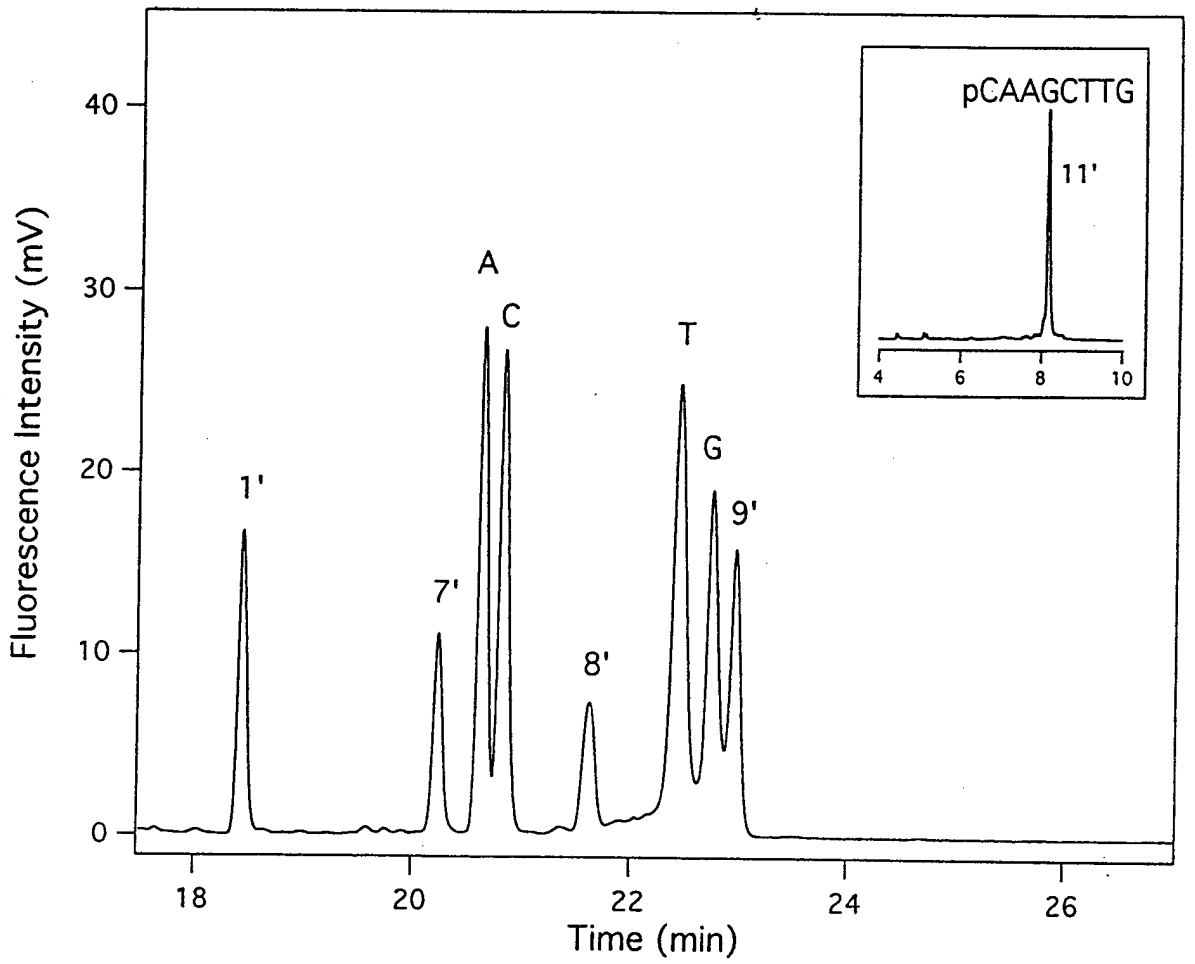


Fig. 4. Electropherogram at pH 10.4 (buffer D) of dihydroxy-BO-IMI conjugates of 5′-dAMP (A), 5′-dCMP (C), TMP (T), 5′-dGMP (G), C8-benzyloxy-5′-dAMP (**7′**), C8-[N-acetyl-N-(2-fluorenyl)]amino-5′-dGMP (**8′**), and C8-hydroxy-5′-dAMP (**9′**). Peak **1′** is dihydroxy-BO-IMI. Inset, corresponding dihydroxy BO-IMI conjugate of the oligodeoxynucleotide 5′-pCAAGCTTG (**11′**), at pH 8.7 (buffer C, which was in use at the outset of our project). Sample preparation: BO-IMI (1.2×10^{-2} *M*), total adducts (1.2×10^{-2} *M*), and EDC (9×10^{-2} *M*), all in buffer A, 10 μl each, were mixed, and the resulting solution was kept unstirred in the dark at room temperature for 2 h followed by filtration through a pasteur pipet column containing ~140 mg propyl sulfonic acid silica retained on glass wool.

failure of this acid to retain, of course, on the cation exchanger. Unfortunately, compound **10′**, in turn, can undergo activation by EDC in the reaction mixture to label amino contaminants. We assume that this accounts for at least some of the other background peaks that are present in this electropherogram. This inherent limitation of BO-IMI, arising from the instability of the leash between the dye and the imidazole moiety, suggests that a leash with greater hydrolytic stability would make it easier to achieve higher sensitivity.

4. Conclusion

BO-IMI is a useful reagent, as demonstrated here (e.g., Figs. 4 and 5) and in our prior publications. While the labeling reactions of phosphomonoesters is complex, the reaction is specific and, overall, can lead to single peaks by CE-LIF from such analytes. We are working on improving the reagent by establishing a more stable leash between the imidazole and dye moieties, towards a goal of extending the sensitivity below the current femtomole level.

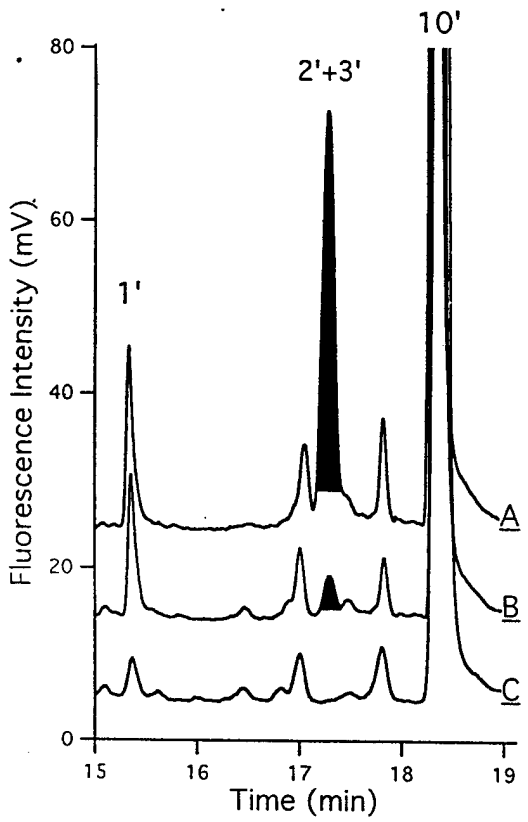


Fig. 5. Electropherograms obtained by reacting 600 (A), 60 (B) and 0 (C) femtomoles of 5'-dAMP with BO-IMI followed by cation exchange filtration, evaporation, addition of buffer D, incubation for 30 min, and injection. Peak assignments: see Table 1.

Acknowledgements

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Submarine Gel Electrophoresis Purification of a Small Molecule: 99.9998% Fluorescently Pure IMI2

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HPLC and related techniques failed to raise the fluorescence purity of a BODIPY–imidazole dye (IMI2) above 99.5%, based on assessment of dye purity by capillary electrophoresis with laser-induced fluorescence detection (CE-LIF). This difficulty was overcome by employing submarine gel electrophoresis, which raised the fluorescence purity level to about 99.9998%. This estimate of final dye purity was made by using immobilized metal affinity chromatography to selectively remove the dye from a sample that was then analyzed by CE-LIF as a 5000× more concentrated sample than the original. The amount of dye purified on a 87- × 67- × 10-mm ($l \times w \times h$) gel in a 40-min run was at the 0.5-mg level. The dye was recovered by soaking the sliced-out electrophoretic band in methanol. We are now in a position to employ this dye for labeling of trace phosphomonoester analytes without being hampered by dye impurities, by further developing methodology that we reported previously (Wang, P.; Giese, R. W. *Anal. Chem.* 1993, *65*, 3518–3520). More generally, we conclude that submarine gel electrophoresis can be a convenient and useful separation technique for purifying small molecules.

Submarine gel electrophoresis is a well-established method for macromolecule separations.[1,2] The basic idea is to conduct the separation in a gel such as agarose under a thin layer of buffer. It is convenient to prepare a gel in this way and also to recover the sample bands after the separation. During the separation, the layer of buffer keeps the gel wet and also helps with cooling. The technique generally is not used with a polyacrylamide gel since the latter requires exclusion of oxygen during preparation.

In this article, we call attention to the method for the purification of small molecules. Several other methods failed to provide the degree of purity that we required for a fluorescent dye, whereas submarine gel electrophoresis solved the problem. The compound that we purified belongs to the class of "IMI dyes" which are useful for labeling phosphomonoesters.[3,4] Dye impurity as a barrier to trace analysis by fluorescence labeling is a general problem.[5]

EXPERIMENTAL SECTION

Equipment. The submarine gel electrophoresis system was model EC370 from E-C Apparatus Corp. (Holbrook, NY). The capillary electrophoresis laser-induced fluorescence (CE-LIF) equipment was described before.[3] A SpeedVac concentrator from Savant Instruments Inc. (Holbrook, NY) was used for vacuum-drying and concentration (without heating). The glassware was used as obtained.

Reagents. Methanol, methylene chloride, tetrahydrofuran, and ethyl acetate were HPLC solvent grade from J.T. Baker. MES buffer (10 mM, pH 6.0) was prepared fresh by combining 475 mL of water, 25 mL of methanol, and 1.066 g of 2-[*N*-morpholino]-ethanesulfonic acid (Sigma, St. Louis, MO) and adjusting the pH with 1 N sodium hydroxide. Any overadjustment was corrected with 1 M sulfuric acid. Sucrose solution was 3 g (Sigma) in 10 mL of MES. Ni-NTA-agarose was from Qiagen (Valencia, CA). Histamine–methanol solution was prepared by combining 50 mg of histamine (no. 53290 from Fluka, Milwaukee, WI) and 100 μL of methanol. Other reagents were obtained as described.[3]

IMI2 Synthesis. BODIPY FL SE (1 mg, Molecular Probes, Eugene, OR) was dissolved in 200 μL of freshly distilled (over sodium) tetrahydrofuran, and 10 μL of histamine–methanol solution was added, giving an immediate precipitate. After 20 min of standing in the dark, the reaction mixture was transferred to a 10-mL test tube, and 1 mL of water was added, dissolving the precipitate. Two portions of 1 mL of freshly distilled methylene chloride were used to extract the product, and these extracts were combined, dried in a SpeedVac, and stored in the dark at 4 °C prior to purification as follows by silica flash chromatography and submarine gel electrophoresis.

Silica Flash Chromatography. A silica column was prepared by packing Silica Gel 60 (particle size 0.040–0.063 mm, EM Science, Gibbstown, NJ) in a 10-mL pipet (up to the 10-mL mark) plugged with glass wool. The mobile phase was 25:75 methanol/ethyl acetate (v/v) with 0.05% triethylamine (prepare 100 mL). After the column was prewashed with 20 mL of mobile phase, the sample was dissolved in 50 μL of methanol, followed by 150 μL of mobile phase and, applied. The major (slower) component was collected and dried in a SpeedVac after the eluent was divided

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into two 2-mL glass vials (no. 60910L with rubber-lined cap from Kimble Glass).

Submarine Gel Electrophoresis. Ultrapure agarose (0.5 g from Gibco, Grand Island, NY) was dissolved in 50 mL of MES buffer with boiling and poured into the four-comb plate after cooling to 60–70 °C (cool enough to handle). The plate was kept at room temperature for 2 h to form the gel, and the comb was removed carefully. The dimensions of the gel were 82 × 67 × 10 mm ($l \times w \times h$). After the gel plate was placed into the apparatus, MES buffer was added to a level 1 mm above the gel. IMI2 (~0.5 mg) was dissolved in 80 μL of methanol, and 200 μL of sucrose solution was added. The sample solution was loaded quickly into the bottom of the four wells, and a current of 50 mA was established by applying 250 V. The electrophoresis was run without cooling for 40 min in the dark, and the voltage was adjusted every 10 min as needed to keep 50 mA. The gel was removed and rinsed with 50 mL of water, and the tailed dye band, which had traveled 6 cm, was sliced out with a water-washed razor blade, giving a 67- × 10- × 10-mm gel slab that was sliced further (20 pieces), placed in a 20-mL disposable scintillation vial (no. 986741 with polypropylene cap from Wheaton), covered with 7 mL of methanol (to extract the dye), and stored at 4 °C in the dark overnight, or for at least 2 h.

IMAC of IMI2 To Assess Impurities. A column was prepared by packing 500 μL of Ni-NTA-agarose gel suspension in a 5.75-in.-long capillary Pasteur pipet plugged with a 0.6-cm^2 piece of Kimwipe (Fisher Scientific, Pittsburgh, PA) and washed with one column volume of 10% methanol. Aqueous sample of IMI2 (see figure caption) was loaded into the bed with air pressure. Elution was done by forcing 2 × 50 μL of 10% methanol through the column with air pressure. The collected sample was concentrated in a SpeedVac to 15 μL (volume measured by uptake into a pipet) before injection of an aliquot into CE-LIF.

Capillary Electrophoresis. CE was performed in a 70-cm-long fused-silica capillary (75 μm i.d., Polymicro Technologies, Inc., Phoenix, AZ) with the detection window 50 cm from the anode injection end. Samples were introduced in the capillary hydrodynamically by raising the anode end 10 cm higher for 20 s. Applying 16 kV to the capillary gave 35 μA. The electrolyte was 10 mM pH 10.6 sodium carbonate buffer, 50 mM sodium dodecyl sulfate.

RESULTS AND DISCUSSION

Detection of trace analytes by labeling with a fluorophore tends to require postderivatization sample cleanup prior to detection in order to overcome interferences. These interferences have three origins: residual fluorophore, contaminants in the fluorophore including fluorophore decomposition products, and fluorescent side products formed in the reaction. With IMI dyes, the first type of interference can be minimized by using immobilized metal affinity chromatography (IMAC) at the end of the labeling reaction to remove residual dye,[6] and the last is reduced by the inherent specificity of the labeling reaction for phosphomonoesters.[3] Thus, we directed our attention, as described here, to the second problem: purifying an IMI dye to a high degree. For this work, we selected IMI2, a dye which incorporates an amide as opposed to the hydrazide linkage in IMI1 (previously named "BO–IMI"),

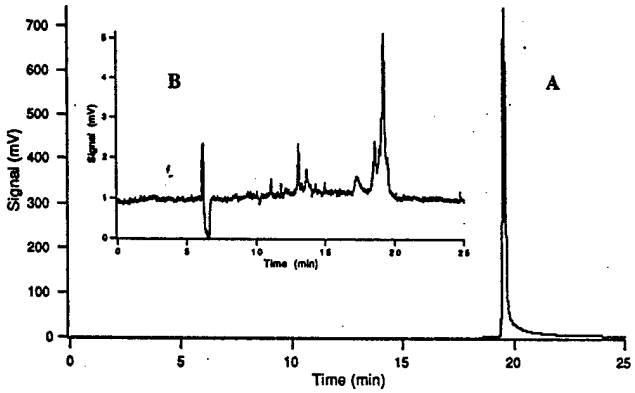

Figure 1. Electropherograms of (A) IMI2 and (B) trace impurities in IMI2. For A, 6.0 × 10^{-5} M IMI2 in water was diluted with water to 8.0 × 10^{-8} M prior to injection. For B, 100 μL of 6.0 × 10^{-5} M IMI2 was subjected to IMAC, vacuum-concentrated to 15 μL, and then injected. IMI2 concentration was determined by UV, using the data established for BODIPY FL SE (Catalog No. D-2184, ϵ = 80 500 at 502 nm in methanol, Lot Data, Molecular Probes, Eugene, OR).

since the hydrazide linkage in the latter compound is susceptible to hydrolysis.[7] No hydrolysis of the amide linkage in IMI2 was observed, even when this dye was kept at room temperature in buffers with pH's ranging from 6 to 10 for 2 days (data not shown).

The starting point for the ultrapurification of IMI2 was dye that had been subjected to silica flash chromatography to reach a purity level in terms of fluorescence of 99.5% based on an analysis by capillary electrophoresis with laser-induced fluorescence detection (CE-LIF). A variety of chromatographic (five types of HPLC columns including mobile phase variations) and partitioning procedures (details not presented), along with immobilized metal affinity chromatography (IMAC), alone and in combination, failed to extend the purity much further. Throughout this work, it was important to minimize exposure of the dye to light and to avoid evaporation to dryness, since these conditions could increase the impurities.

We decided to try preparative electrophoresis for purification as a more orthogonal technique relative to the ones already tried, and we selected a submarine gel for this purpose because of its convenience. Indeed, we estimated that a fluorescence purity of 99.9998% was achieved for IMI2 in this way. The data leading to this conclusion are shown in Figure 1. We subjected one aliquot of this reagent directly to CE-LIF (Figure 1A, peak area 1.343 × 10^9) and a second aliquot first to IMAC (to selectively remove the dye) prior to evaporative concentration followed by CE-LIF (Figure 1B). In a separate experiment, we observed that IMAC alone had a negligible impact on the purity of IMI2 at this stage (data not shown). Thus, the observed impurities are enriched 5000 times in A relative to B. We arbitrarily assumed that the observable impurities (Figure 1B; peak areas sum to 2689) had a fluorescent brightness equivalent to that of IMI2 in estimating the fluorescence purity level. One of the peaks (e.g., the tallest one) in Figure 1B may be residual IMI2, in which case the fluorescent purity of the dye is higher than 99.9998%. Presumably, the level of nonfluorescent impurities is low, given the multiple purification steps and the high fluorescence purity. Consistent with this, the dye gives a single spot by silica TLC with UV quench

(6) Shen, X.; Giese, R. W. *J. Chromatogr.* **1997**, *777*, 261–265.

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detection (developed with methanol/CH_2Cl_2, 1:10, v/v), in fact, both before and after the electrophoresis purification step.

CONCLUSION

Submarine gel electrophoresis can be attractive as a secondary purification technique when other methods fail. We found this technique to be convenient, relatively low in cost (both in terms of equipment and ingredients), and powerful for ultrapurifying a fluorescent dye. Obviously, the convenience of the method is reduced when the substance of interest lacks fluorescence or color. Nevertheless, detection by UV shadowing or some other technique can be used in other cases.[8] This includes the option to remove and stain (or analyze in some other way) a thin, longitudinal slice of the gel. Though not demonstrated here, the method can be applied to nonionic compounds by including micelles in the gel. Finally, one can anticipate excellent recoveries of small compounds in general due to the weak adsorption properties of an agarose gel.

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Short communication

Preparation of an IMI dye (imidazole functional group) containing a 4-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole fluorophore for labeling of phosphomonoesters

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Abstract

We are studying dye–imidazole conjugates ("IMI dyes") as reagents for labeling phosphomonoesters such as nucleotides. Previously we have employed a BODIPY dye in our IMI reagents, and analyzed the labeled products by capillary electrophoresis with laser-induced fluorescence detection (CE–LIF) involving an argon ion laser. (The BODIPY fluorophore is a 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene). Here we broaden the technology by preparing a DBD–IMI dye [DBD=4-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole], and using a helium–cadmium laser. While DBD–IMI (IMI3) is about 50× more stable photolytically than a BODIPY–IMI dye (IMI2, a conjugate of a BODIPY dye with histamine, was tested), the detection limit for IMI2 ($5 \cdot 10^{-11}$ *M*; *S/N*=5, CE–LIF with an argon ion laser) is tenfold better than that for IMI3 ($5 \cdot 10^{-10}$ *M*, *S/N*=5, helium–cadmium laser). IMI3 conjugates of the four major DNA nucleotides were prepared and detected by CE–LIF.

Keywords: Detection, electrophoresis; Derivatization, electrophoresis; Dyes; Nucleotides

1. Introduction

Previously we introduced fluorescent dye–imidazole conjugates ("IMI dyes") as derivatization reagents for the detection of phosphomonoesters [1–4]. These dyes incorporate an imidazole reactive group which covalently links to a phosphomonoester once the latter is activated with a carbodiimide reagent. The dye-labeled phosphomonoester then can be detected based on its fluorescence, e.g., by means of capillary electrophoresis with laser-induced fluorescence detection (CE–LIF). In this way we have detected standards of some nucleotides, glucose-6-phosphate, and *O*-phosphotyrosine. Glucose-1-phosphate and *O*-phosphoserine did not label apparently due to electrostatic or steric effects [1]. Also we have detected pepsin (a phosphoprotein) via phosphate-specific labeling with an IMI tag [2]. The labeling reaction is specific for phosphomonoesters because the side reaction with carboxylic acids leads to *N*-acylimidazoles which are hydrolytically unstable. Mechanistic details of the reaction were studied [3], and a slight hydrolytic instability of IMI1 (a conjugate of BODIPY FL C_3 hydrazide with N-acetylhistidine that we originally called "BO–IMI [1]) was overcome by the preparation of IMI2, a conjugate of

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BODIPY FL SE with histamine [4]. (The BODIPY fluorophore is a 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene).

Thus far our IMI reagents have incorporated a BODIPY dye, which has an absorption maximum near the 488 nm line of the argon ion laser. This is the type of laser that we have employed to date in our CE–LIF system. Since the helium–cadmium laser also is employed frequently in CE–LIF systems, we sought to develop an IMI dye suitable for this alternative equipment.

We have selected a DBD dye for this purpose, comprising a 4-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD) fluorophore. This type of dye was introduced and further developed by Imai and coworkers (e.g., Refs. [5,6]) in the form of chiral reagents for the analysis of various racemates by derivatization–HPLC–fluorescence detection. An excitation wavelength of 450 nm was employed [6] which is close to the 442 nm line of a helium–cadmium laser. Conveniently, the dye is available commercially as an acid chloride, suitable for conversion to an IMI reagent. Two additional features that made us select this dye are its large Stokes shift of about 120 nm, and its lack of functional groups that could react with the carbodiimide and thereby complicate our labeling reaction for a phosphomonoester.

2. Experimental

2.1. Chemicals

4-(*N*,*N*-Dimethylaminosulfonyl)-7-(2-chloro-formylpyrrolidin-1-yl)-2,1,3-benzoxadiazole [(*R*)-(+)-DBD-ProCl] was purchased from Tokyo Kasei (Tokyo, Japan). Histamine was from Fluka (Buchs, Switzerland) and was recrystallized in chloroform before use. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC), 2-(*N*-morpholino)ethanesulfonic acid (MES) and the sodium salts of 2′-deoxyadenosine 5′-monophosphate, thymidine 5′-monophosphate, 2′-deoxycytidine 5′-monophosphate, and 2′-deoxyguanosine 5′-monophosphate (dGMP) were from Sigma (St. Louis, MO, USA). Electrophoresis purity grade sodium dodecyl sulfate (SDS) was purchased from Bio-Rad (Hercules, CA, USA). ACS grade sodium bicarbonate was from Baker (Phillipsburg, NJ, USA). Distilled deionized water was used in the preparation of all aqueous solutions. All other chemicals and organic solvents were of analytical-reagent grade and were used without further purification. IMI2 was prepared as described previously [4].

2.1.1. DBD–Pro-IMI (IMI3)

To a 1 ml chloroform solution of (*R*)-(+)-DBD-ProCl (2.23 mg, 6.22 μmol) was added 1.5 ml of chloroform containing histamine (1.73 mg, 15.6 μmol) and the mixture was stirred at room temperature overnight. The residue from evaporation was subjected to silica flash chromatography (10 ml pipet column) using methanol: chloroform (5:95, v/v) as mobile phase. The fraction containing the product (second band) was dried in a Savant SpeedVac concentrator (Holbrook, NY, USA) and then was further purified by submarine gel electrophoresis using the technique that we have described previously [3]. Matrix assisted laser desorption ionization time-of-flight mass spectrometry was used to confirm the molecular mass of the product, with 2,4,6-trihydroxyacetophenone as the matrix.

2.2. Nucleotide labeling with IMI3

IMI3 was concentrated to $5 \cdot 10^{-4}$ *M* in a Savant SpeedVac concentrator. Nucleotides (10^{-6} *M*) and EDAC (0.10 *M*) were prepared in 0.10 *M* pH 7.0 (adjusted with NaOH) MES buffer. Five μl aliquots each of the nucleotide mixture, EDAC, and IMI3 were added to a glass conical vial and then mixed thoroughly by swirling. The reaction was allowed to stand in the dark overnight. An aliquot of 1.5 μl was removed and subjected to immobilized metal affinity chromatography (IMAC) as described before [4].

2.3. CE–LIF

The CE–LIF system was the same as that reported before [1], except that a different laser, wavelength, and set of filters were used: helium–cadmium laser (Liconix, Santa Clara, CA, USA), 14 mW, 442 nm excitation, 560 nm detection; 442 nm laser line filter/560 nm interference filter (Oriel, Stratford, CT, USA). The separation was done in a 65 cm (40 cm to

detector)×75 μm I.D. capillary at 16 kV (35 μA) with anodic siphoning injection (anode elevated 10 cm for 20 s to load about 10 nl). The CE buffer was 10 m*M* pH 10.60 sodium carbonate with 50 m*M* SDS.

2.4. Exposure to UV

A solution of IMI3 (100 μl of $3.7 \cdot 10^{-4}$ *M*) in methanol was placed in a conical glass vial and placed 1.5 cm under a spectraline Model ENF-24 UV lamp (365 nm) from Spectronics (Westburn, NY, USA) for 30 min at room temperature. After IMAC was done as described previously [4], the resulting sample (50 μl, 10% methanol) was subjected to CE–LIF. A control sample was processed in the same way, except for the UV irradiation. Also corresponding samples and controls of IMI2 were treated in the same way, except that the initial samples were 50 μl of $5.8 \cdot 10^{-5}$ *M* IMI2.

3. Results and discussion

IMI3, the structure of which is shown in Fig. 1, was prepared by reacting histamine with *R*-(+)-DBD-Pro-COCl. The reaction conditions were the same as what we used previously to prepare IMI2 [4]. It is conceivable that an *N*-acylimidazole intermediate might have formed in the reaction and then reacted further to form the desired product, but we did not study this. During the subsequent purification step by silica flash chromatography, there was one, unidentified colored band that moved faster than the analyte. The collected product gave a single, colored band during the subsequent purification step by submarine gel electrophoresis, which was conducted as described before [4].

The detection of $5 \cdot 10^{-10}$ *M* IMI3 by CE–LIF with a helium–cadmium laser is shown in Fig. 1, where the *S/N* is about 5. A similar *S/N* is observed for $5 \cdot 10^{-11}$ *M* IMI2 by CE–LIF with an argon ion laser (data not shown). Thus IMI3 is tenfold less sensitive

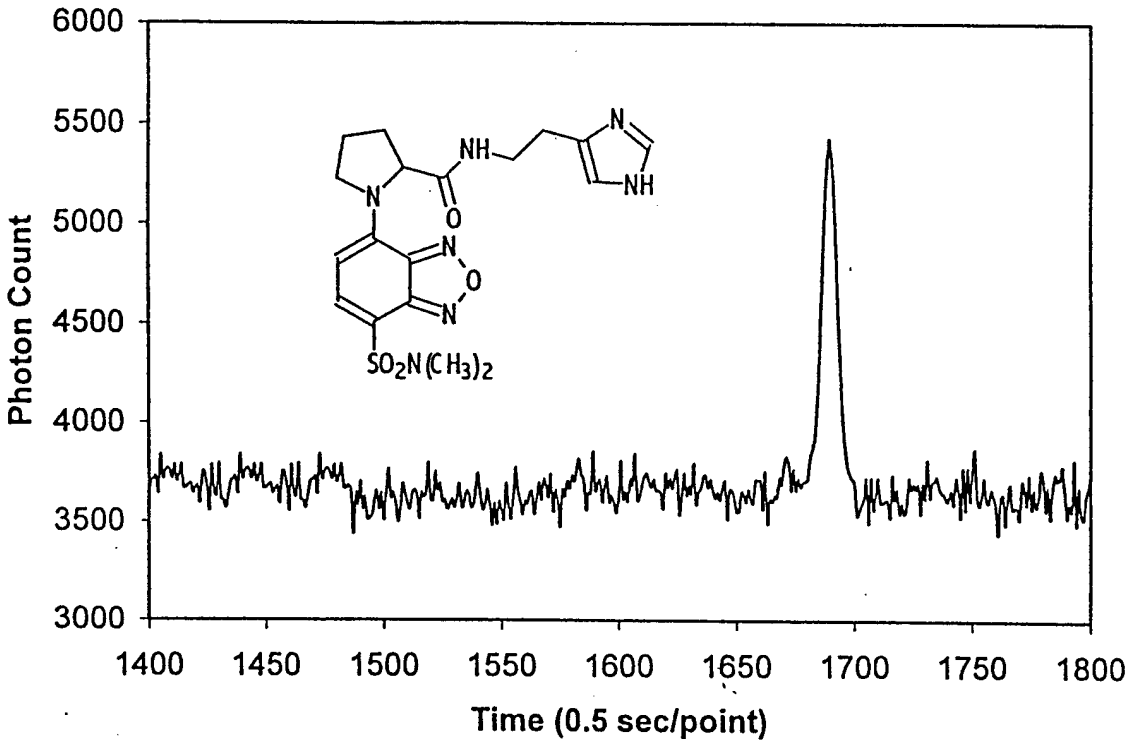

Fig. 1. Structure of IMI3, and detection of a $5 \cdot 10^{-10}$ *M* sample of this reagent by CE–LIF with a helium–cadmium laser. Electrolyte: 10 m*M* pH 10.60 sodium carbonate with 50 m*M* SDS.

than IMI2 in this comparison, which involves an optimum excitation wavelength for each. When IMI3 is measured with an argon ion laser, which is not optimum for it, a similar S/N is observed for $1 \cdot 10^{-8}$ M.

It is well known that organic compounds in general, and especially fluorophores, tend to degrade when exposed to light. Some fluorophores seem to be more susceptible than others to such degradation. We decided to compare IMI2 and IMI3 in this respect, using conditions relevant to our analytical procedure. The dye moiety in IMI2 is BODIPY whereas it is DBD in IMI3.

We exposed methanol solutions of IMI2 ($5.8 \cdot 10^{-5}$ M) and IMI3 ($3.7 \cdot 10^{-4}$ M) to UV radiation (365 nm) for 30 min. (A higher concentration of IMI3 was used since its fluorescence signal is about tenfold lower than that of IMI2; as just pointed out.) After the samples were subjected to IMAC, the electropherograms shown in Fig. 2 were obtained. Residual IMI3 is seen in both the exposed (a) and nonexposed (b) samples of this dye (Fig. 2B), apparently because its higher concentration prevented its complete removal on the small IMAC column employed. Taking into account the 13-fold higher concentration of the IMI3-derived (B) vs.

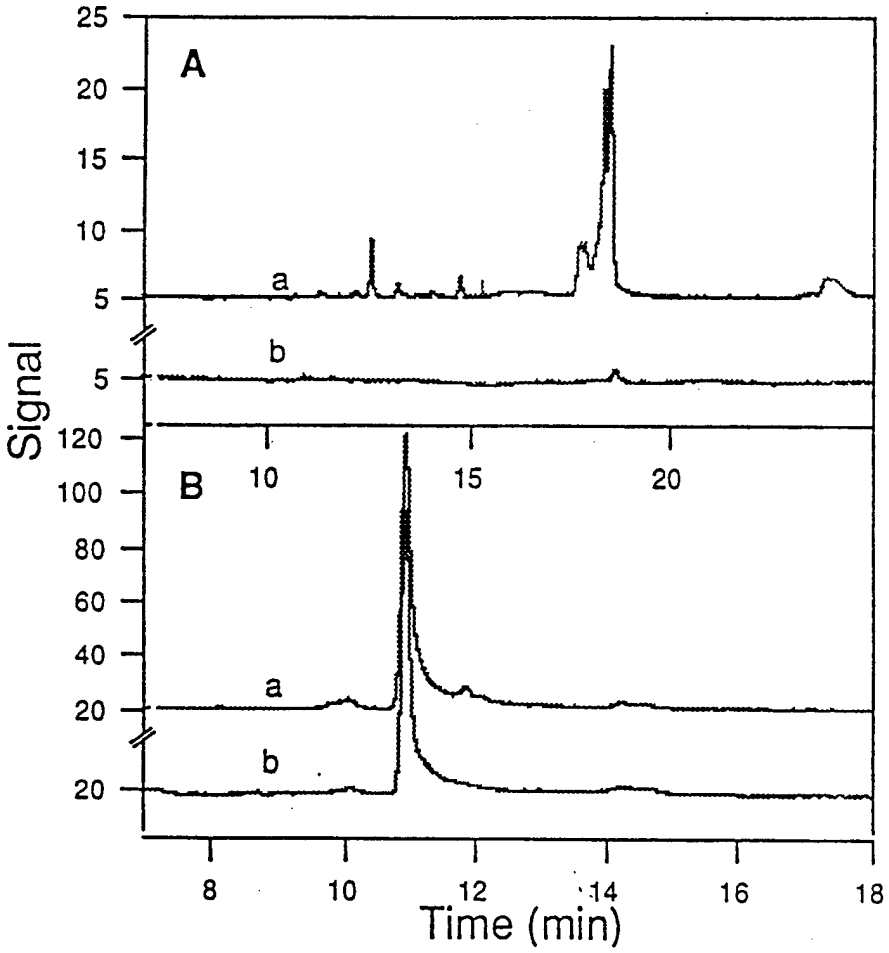

Fig. 2. Electropherograms of IMI2 (*A*) and IMI3 (*B*) before (b) and after (a) exposure to UV radiation followed by elution through an IMAC column. The concentration of the final sample is 13× higher in B than in A.

IMI2-derived (A) samples that are analyzed in Fig. 2, we see that the level of fluorescent impurities not retained on IMAC is about 50-fold higher in the IMI2-derived sample. Assuming that these impurities have undergone photolytic damage to the imidazole moiety (because they are unretained on IMAC), then the microenvironment of the imidazole moiety is quite different in the two dyes. Perhaps the richer array of functional groups in DBD relative to BODIPY causes a tighter intramolecular interaction of the dye and imidazole moieties in IMI3 and this reduces photolytic damage to the imidazole in this compound. On the other hand, the dye moieties might differ in the degrees to which they are causative rather than protective, since imidazole can be oxidized upon exposure to light in the presence of a photosensitizing dye [7]. Obviously the true mechanism is totally obscure.

Reaction of IMI3 with a mixture of the four, normal nucleotides as 5′-monophosphates, leads, after IMAC, to the electropherogram shown in Fig. 3. The separation mechanism is complex, in part because SDS micelles are present, but the effective mobility of IMI3–dGMP may be the highest mainly because guanine has the lowest acidic pK_a of the four DNA nucleobases. A comparable experiment with IMI2 (data not shown) gives about tenfold higher sensitivity, consistent with the above comparison of the IMI2 and IMI3 dyes.

4. Conclusion

The technique of labeling a phosphomonoester with an IMI dye and detecting the products by CE–LIF has been extended to a CE system incorporating a helium–cadmium laser. This was accomplished by preparing IMI3, which incorporates a DBD dye. Relative to IMI2, which incorporates a BODIPY dye, IMI3 affords tenfold poorer sensitivity

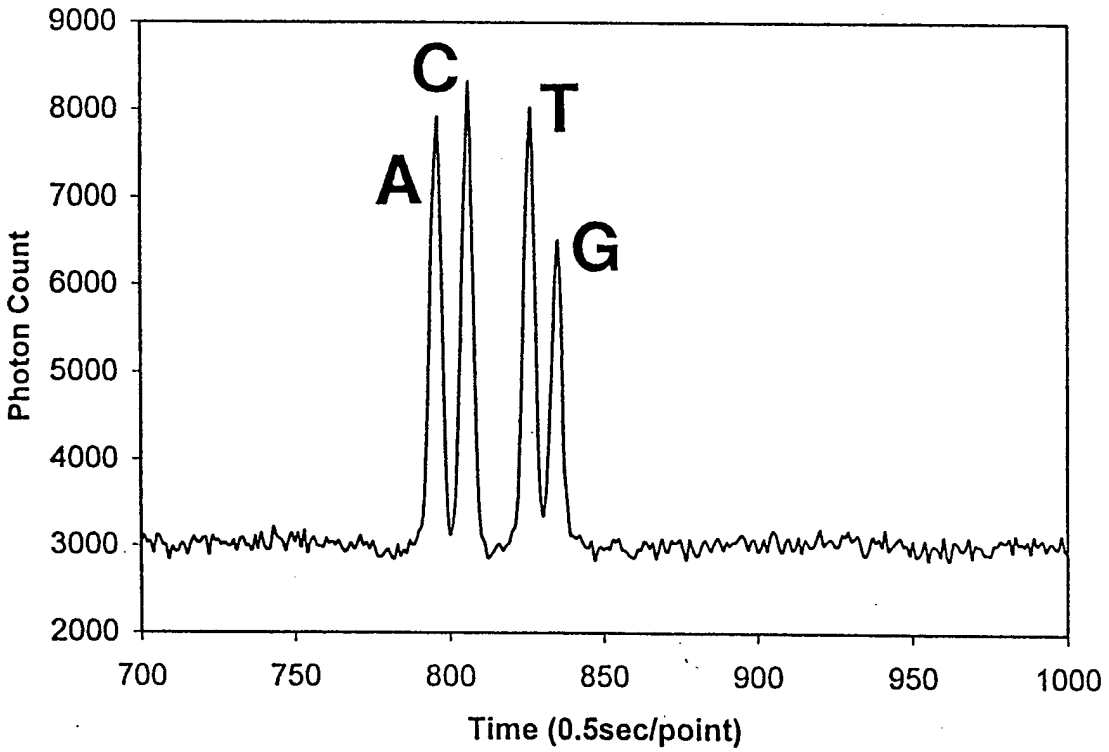

Fig. 3. Separation of IMI3 conjugates of nucleoside 5′-monophosphates by CE–LIF using the same conditions as in Fig. 1.

(argon ion laser for IMI2, helium–cadmium for IMI3, which is optimal for each).

Acknowledgements

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Fluorescence/MS Detection of DNA Adducts

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There is much evidence that DNA adducts (covalent damage to DNA) can initiate cancer, so it is important to measure them in humans. Much has been accomplished in this area by ^{32}P-postlabeling, but many of the detected adducts continue to be unknowns, and it is unclear what fraction of the total adducts are being detected.

Fluorescent dye labeling of DNA adducts potentially can help to fill in these gaps, due to the high performance of capillary electrophoresis with laser induced fluorescence detection (CE-LIF), and the compatibility of the labeled products with detection by mass spectrometry. We are developing IMI dyes (imidazole is the functional group) for this purpose, which achieve phosphate-specific labeling via a water-soluble carbodiimide reaction (1-3). We have detected diluted standards of BO-IMI labeled nucleotides at the low attomole level (polarity switching injection of 4.4 µL containing 2.2 amol of a BO-IMI nucleotide) by CE-LIF), as described before (1).

In Figure 1 is shown our recent detection of benzo[a]pyrene diolepoxide DNA adducts of deoxyadenosine-5'-monophosphate from a test tube experiment. As seen, the sample was analyzed by CE-LIF under both micellar electrokinetic chromatography (MEKC) and non-MEKC conditions (electropherograms in Fig. 1A and 1B, respectively).

In Figure 2 is shown the MALDI mass spectrum of a BO-IMI labeled benzo[a]pyrene diolepoxide adduct of guanine. The amount of the adduct in the laser beam is about 300 fmol and a high signal to noise is observed. Not shown is a high resolution analysis of the molecular ion in which the isotopic distribution fits that calculated for this species.

Thus, fluorescence post-labeling with IMI dyes involving analysis by both CE-LIF and MALDI-MS promises to advance our knowledge both qualitatively and quantitatively about the range of unknown DNA adducts present in human samples.

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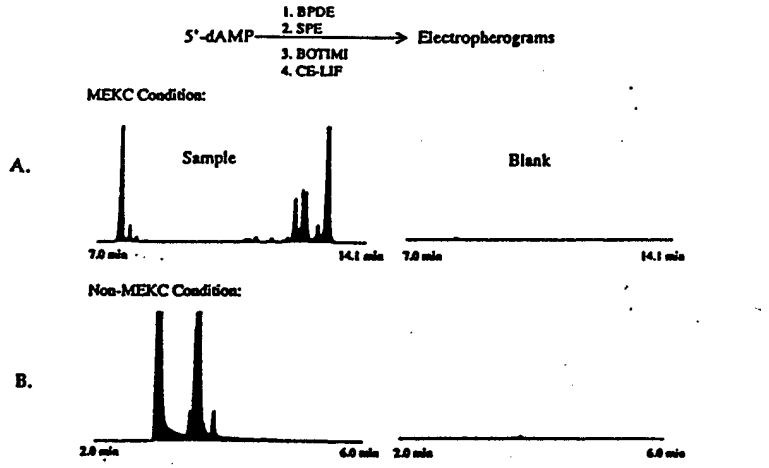

Fig. 1. Electropherograms of products from the reaction of 5'-dAMP with benzo[a]pyrene-r-7, t-8-dihydrodiol-t-9,10 epoxide (±), (anti)

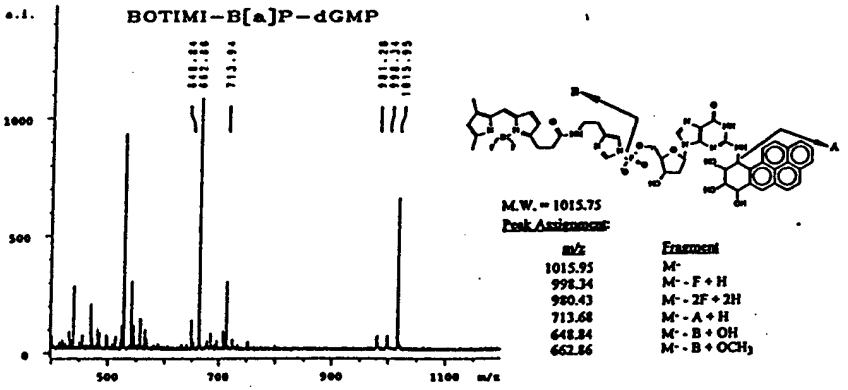

Fig. 2 Detection of an IMI-labeled DNA adduct standard by MALDI-MS.

Proceedings of the 46th ASMS Conference on Mass Spectrometry and Allied Topics, May 31-June 4, 1998, Orlando, FL

DETECTION OF DNA ADDUCTS IN HUMAN BREAST TISSUE

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Introduction. It is likely that DNA adducts initiate breast cancer and they may contribute as well to its progression. Current methods to measure DNA adducts in breast tissue are limited. We propose in this project to set up and apply powerful new analytical methodology for the detection of both known and unknown DNA adducts in human breast tissue. The hypothesis to be tested in this project is that nonpolar DNA adducts explain the epidemic of breast cancer. Because of the high content of fat in breast tissue, nonpolar chemicals may accumulate there. Risk factors may act by causing or promoting the occurrence of DNA adducts that in turn initiate or promote breast cancer. This role of risk factors potentially can be revealed by correlating them with DNA adducts in breast tissue. In turn, this may require comprehensive and accurate measurement of DNA adducts in such tissue. While this cannot be done at the present time, we intend to reach a first stage towards such methodology in this project: the general measurement of many nonpolar DNA adducts, both alkyl and aromatic, in breast and, for comparison purposes, in other tissues as well.

The concept for our proposed new methodology for measuring DNA adducts is the same as ^{32}P-postlabeling, but the details are quite different. By changing the details, we intend to overcome the following limitations of ^{32}P-postlabeling: dependence of labeling on adduct structure, limited resolution, different conditions for different adducts, and the radiolabel barrier to analyzing the adducts by mass spectrometry.

Experimental Procedures. In our proposed method, DNA is isolated from the tissue and digested to deoxynucleotides using conventional techniques. The deoxynucleotides are then separated by HPLC and fractions are collected. The deoxynucleotides in each

Keywords: DNA Adducts, Fluorescence Labeling, Capillary Electrophoresis, Breast Tissue

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fraction are fluorescence-labeled on their phosphate group with a BO-IMI fluorescent dye that we have synthesized, followed by capillary electrophoresis with laser-induced fluorescence detection (CE-LIF). This dye specifically labels phosphomonoesters under aqueous conditions at room temperature.

Results. To facilitate the purification of our BO-IMI dye, and also removal of excess dye after the labeling reaction, we have studied its separation, along with that of a precursor dye, BODIPY-hydrazide, by immobilized metal affinity chromatography (IMAC). The two compounds were separated on three forms of a Sepharose-IDA column: Cu(II), Ni(II) and Zn(II). Whereas BO-IMI eluted first on the Cu(II) and Ni(II) columns (a pH gradient from 7.0 to 2.0 was applied), it eluted last on the Zn(II) column. BO-HZ eluted from the Zn(II) column without displacing this metal. The explanation suggested for these results is that BODIPY hydrazide undergoes strong, bidendate binding only to the Cu(II) and Ni(II) columns.

We have also studied, for labeling of standard nucleotides, the sensitivity of our BO-IMI labeling/CE-LIF technique. To date, we have been able to detect 50 femtomoles of thymidine-5'-monophosphate by directly injecting a labeling reaction containing this amount of analyte into CE-LIF. Also, for a diluted, pure standard, polarity switching injection enables a low attomole amount of analyte in a practical sample volume to be detected.

Conclusion. Early results on our new methodology indicate that it will be convenient and provide high sensitivity for the analysis of DNA adducts derived from human breast samples.